AN *IN VITRO* INVESTIGATION OF THE EFFECT OF KHELLIN AND UVA (KUVA) ON NORMAL AND TRANFORMED HUMAN MELANOCYTES

GADIJA CARLIE

Submitted in fulfillment of the requirements for the degree of

MAGISTER SCIENTIAE

in the

FACULTY OF MEDICINE

at the

UNIVERSITY OF CAPE TOWN

October 1999

Supervisor: **Prof. S.H. Kidson**

Co-supervisor: **Dr. P. Hulley**

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October, 1999

ACKNOWLEDGEMENTS:

In deep gratitude, I would like to thank the following people who made this thesis possible:

Professor S.H. Kidson, my supervisor, for her invaluable guidance, constant nurturing and supervision

Dr Phillipa Hulley, my co-supervisor, for her excellent guidance, academic and emotional support, and encouragement when the end was far from sight

Dr Ebrahiem (surgeon) and nursing staff at Gatesville Medical Centre for the supply of neonatal skin samples for culturing normal human melanocytes

Dr Dommisse (plastic surgeon) and nursing staff at Claremont Hospital for the supply of skin samples for melanocyte cultures

Dr N.A. Parker (general practitioner) for neonatal skin samples also supplied

Mr G. Hanekom, Dept. of Clinical Science and Immunology (UCT) for the supply of the human melanoma cell line (UCT Mel-1)

Mr Craig and the staff of the Dept. of Clinical Engineering (UCT, Groote Schuur Hospital) for making the UV cabinet

Professor Vincent Hearing, National Institute of Health (United States of America) for the provision of antibodies for western blot analyses

Ms Sharon Prince for critical reading of the thesis and also for her advice and friendship

Mrs Toni Wiggins for her efficient technical support with western blot analyses. In addition, her friendship, advice and constant support

Mr Mike Butterworth for his assistance with the scanning of the blots

Mr Henry Fortuin for his assistance with the photography

The MRC, Smithkline Beckman and UCT for financial assistance during the course of this project

My mom and dad for their constant support and encouragement while being a student

The Parker family, especially my new mom, for their support and understanding

My husband and best friend, Hosain, for his constant understanding, motivation and undying support

All those not specifically acknowledged for their assistance in the completion of this project

DEDICATION

Firstly, to **Hosain**, with love and

Secondly, to the **vitiligo sufferer** who wrote this letter and all those sufferers who feel this way:

"Many people seem to believe that it shouldn't bother me because it isn't painful. Sure it isn't painful, but it certainly is doing a job on me mentally. Well, I have had vitiligo almost half my life and to be honest I feel as though life stopped somewhere around age 23 or so for me. This is when it started getting bad. Since then I have been in a sort of limbo waiting for a cure to take place. I am not enjoying life the way it was meant to be. I am simply existing, waiting for my cure so I can catch up with and join the rest of the beautiful people. I may sound bitter about this and maybe I am. I don't recall doing anything bad enough to deserve this, and why has it been decided for me to have instead of you anyway? Why did I get it now instead of when I got real old and wouldn't care? People just expect me to accept it and continue on. I get these disgusted looks, as if "Here she comes, the walking, talking horror show". I feel I should join the circus as one of their freak acts. They have the snake man, an albino lady, a fat lady, now what they need is the bleach lady. I feel like "Casper the friendly ghost". All I want is to be friends, but the sight of me makes people feel ill at ease, very uncomfortable with me.

When the doctor asked me what vitiligo means to me, my first answer was that I feel like a mistake. If it isn't a mistake then all you one-colored persons are the mistake. I don't see all you one-colored persons trying to get bleached out till you're two colors. So this shows that it is just that: I am a mistake! I believe if there were no hope for me I would crack up, but if I were lucky I would end it all first. They say where there is life there is hope. The doctors say they will treat me only as long as I am repigmenting. At least fully dressed, with long pants and long-sleeved shirts, I look almost like one of you humans. To be rid of vitiligo would be like being reborn for me, to be normal and happy."

(A letter from an anonymous vitiligo sufferer published in Clin. Dermatol.; 1997; 15: 886)

TABLE OF CONTENTS: **PAGE:**

Deals with the:

- **Dissolution of khellin**
- **Effect of khellin treatment on the proliferation of Mel-1 melanoma cells, normal human melanocytes and 3T3 fibroblasts**
- **Establishment of normal melanocyte cultures**
- **Effect of UVA treatment on the proliferation of Mel-1 melanoma cells, normal human melanocytes and 3T3 fibroblasts**
- **Effect of the combination of khellin and UVA (KUVA) treatment on the proliferation of Mel-1 melanoma cells, normal human melanocytes and 3T3 fibroblasts**

LIST OF FIGURES

LIST OF ABBREVIATIONS:

UV :	Ultraviolet light. UVA= ultraviolet A light, UVB= ultraviolet B light
KUVA:	The drug khellin is combined with UVA light
PUVA :	The drug psoralen is combined with UVA light
MSH :	Melanocyte-stimulating hormone
TRP-1 :	Tyrosine-related protein-1
TRP-2 :	Tyrosine-related protein-2
DOPA:	3,4-dihydroxy-phenylalanine
DAG(s):	Diacylglycerol(s)
cGMP :	Guanosine 3,5-cyclic monophosphate
PKC :	Protein Kinase C
PKA :	Protein Kinase A
MAP Kinase:	Mitogen Activated Protein Kinase
7-BH_4 :	7-tetrahydrobiopterin
8-MOP:	8-methoxypsoralen
EDTA :	Ethylenediamine tetraacetic acid
PBS :	Phosphate-buffered saline
TPA :	12-O-tetradecanoyl-phorbol-13-acetate
IBMX :	Isomethylbutylxanthine
DMSO:	Di-methylsulfonamide
SDS :	Sodium dodecyl sulphate
TBS :	Tris buffered saline
PMSF :	Phenyl methylsulfonyl-flouride
TCA :	Trichloroacetic acid
NHMs :	Normal human melanocytes

ABSTRACT

An *in vitro* investigation of the effect of KUVA (Khellin and UVA) on Normal and Transformed Human Melanocytes

Gadija Carlie
Department of Anatomy and Cell Biology, Medical School, University of Cape Town, Observatory 7925
Cape Town, South Africa
October 1999

The problematic and numerous side effects of PUVA (psoralen and UVA) and other treatments currently in use for vitiligo, have justified the search for an alternative treatment. In this study, the use of khellin, a naturally occurring furochromone, which is similar in structure to psoralens, is explored as an alternative treatment for vitiligo. When khellin is combined with UVA (KUVA), it is reported to repigment vitiligo skin as effectively as PUVA photochemotherapy, but without the adverse effects reported with PUVA. The exact mechanism for khellin-induced repigmentation is still to be determined and no cell biological studies have yet been done to elucidate its mechanism of action. The specific aim of this project was to set up an *in vitro* tissue culture system to determine the direct effects of khellin, UVA and KUVA on melanocyte proliferation and pigmentation.

Studies were carried out on cultures of a human melanoma cell line (Mel-1), normal human melanocytes and 3T3 mouse fibroblasts. Cell proliferation assays revealed that the proliferation of melanoma cells and melanocytes were increased after exposure to khellin for four days at concentrations ranging from 1nM to 0.5mM. A peak of proliferation was obtained at 0.01mM khellin, which stimulated proliferation of melanoma cells and melanocytes by 2.35-fold and 2.1-fold, respectively. In contrast, khellin decreased proliferation of fibroblasts over the entire concentration range tested. At concentrations of 0.5mM and above, khellin was cytotoxic to both melanocytic cells and fibroblasts. Cytotoxic assays revealed that 1mM khellin was equally cytotoxic to both melanoma cells and fibroblasts. In addition, these assays revealed that the proliferative response observed with 0.01mM and 0.1mM khellin, did not mask an underlying cytotoxic effect. Exposure to single doses of UVA between 150-280mJ/cm^2, increased proliferation of melanoma cells with maximal proliferation at 250mJ/cm^2, while the proliferation of normal

melanocytes and fibroblasts were unaffected by this UVA dose. More significantly than khellin or UVA alone, the treatment with the combination of khellin and UVA (KUVA) stimulated proliferation of the melanocytic cells. The combination of 0.01mM khellin plus a single dose of UVA at 250mJ/cm^2 was the most effective treatment. KUVA combination treatments were found to be more cytotoxic to the fibroblasts than khellin or UVA alone.

To test the effect of khellin, UVA and KUVA on melanogenesis, standard radiometric assays were carried out. The combination of khellin and UVA enhanced melanogenesis of the melanocytic cells more significantly than khellin alone or UVA alone. The dose of 0.01mM khellin plus 250mJ/cm^2 UVA (maximal proliferative dose) increased melanogenesis of the melanocytes by 290% above the untreated control melanocytes.

To determine whether khellin and KUVA act by increasing levels of melanogenic proteins, western blot analyses were carried out. The results revealed that there were no differences in the amounts of TRP-2 and tyrosinase in the melanocytic cells treated with khellin alone. In contrast, those treated with KUVA (or UVA alone) had increased levels of the glycosylated form of the enzymes and also possibly had increased levels of the *de novo* form of the enzymes. These results suggest that UVA might be enhancing glycosylation of melanogenic enzymes and provides a novel insight into the possible mechanism for UVA-induced melanogenesis. The results also revealed that 3T3 fibroblasts expressed the non-glycosylated form of TRP-2, as described by others.

In conclusion, this study suggests a model in which khellin acts directly on melanocytic cells by acting as a mitogen and a melanogen at non-toxic concentrations. Khellin possibly increases melanogenesis by acting post-translationally or antagonizing or removing an inhibitor of the melanogenic pathway. The melanocyte-specific effect of khellin and even more so KUVA, seems to suggest that khellin acts along the signal transduction pathways which increases both proliferation and melanogenesis in melanocytes, possibly via endothelin-1 pathways.

Key words: **vitiligo/ khellin/ KUVA/ ultraviolet A/ cell proliferation/ melanogenesis/ western blotting/ normal human melanocytes/ melanoma cells/ melanin formation assays**

CHAPTER 1

1.1 INTRODUCTION:

Vitiligo is a common hypopigmentary disorder of the skin and sometimes hair, with serious psychological and social consequences (Kent and Al' Abdie, 1996 a, b). Vitiligo affects approximately 1 to 5% of the world's population (average of 1 in 200 individuals), and incidence levels are reported to be as high as 8% in India and Pakistan (Nordlund, 1997; Schwartz and Janniger, 1997). This disease may occur at any age and affects both male and female sex equally. It is not certain whether vitiligo is a genetic disease. However, it is known that the chance of developing vitiligo is 4-5 times higher in those who have close biological relatives who suffer from this disease, compared to the general population (Das et. al., 1985). Furthermore, it has been shown that vitiligo is linked to several genes, but the inheritance pattern of this disease did not follow the typical Mendelian genetics or X-linked patterns (Mehta et. al., 1973; Hafez et. al., 1983; Majumder et. al., 1993; Nath et. al., 1994)

The aetiology of this disease is still unknown. What is known, is that the melanocytes (pigment-producing cells) in the epidermis of the skin, progressively disappear until they are completely absent in the vitiliginous lesions, as shown by histochemical (Le Poole et. al., 1992; 1993 a, b; 1996) and ultrastructural studies (Galardi et. al., 1993; Bartosik et. al., 1998). This results in the milk-white blotches on the skin, which gradually become larger as the disease progresses. Besides the melanocytes being progressively destroyed, the adjacent epidermal cells may also be somewhat altered (Bhawan et. al., 1993; Le Poole et. al., 1996), by an unknown mechanism.

There are several treatments for vitiligo currently in use (see reviews Schwartz and Janniger, 1997 and Kovacs, 1998), but the problematic and numerous side effects associated with these treatments have justified the search for an alternative treatment for vitiligo. In this study, the use of khellin as an alternative treatment for vitiligo is explored. Since melanocytes are predominantly affected in this disease, the focus of this study was to determine the effect of khellin on melanocyte proliferation and pigment synthesis (melanogenesis). The following review includes a general overview of the current understanding of the origin, development and biological functions of the melanocytes, as well as a description of vitiligo pathology, vitiligo aetiology, mechanisms and shortcomings of current vitiligo treatments.

1.2 FUNCTIONS OF MELANIN

The most important function of melanocytes is the synthesis of melanin, which protects the skin and underlying organs from photo-damage and photo-carcinogenesis (reviewed Diffey et. al., 1998). This protective function is brought about by melanin acting as a scavenger of toxic oxygen radicals and cytotoxic agents such as amines and metal ions generated within the melanocyte cytoplasm (see reviews Gilchrest, 1996; Applegate and Frenk, 1995, Memoli et. al., 1997). (Figure 1.1) In addition, melanin in the skin and hair provides the colour, which serves as a protective colourant and sexual attractant within species. Besides the melanin itself having functions, it has also been shown that melanogenic intermediates play an important role in many physiological and pathological conditions, such as tissue repair/ regeneration, immune and inflammatory processes (Prunieras, 1986; Nordlund et. al., 1988; Slominski et. al., 1993). The indole melanogenic intermediates, DHI and DHICA (5,6-dihydroxyindole and 5,6-dihydroxyindole-2-carboxylic acid, respectively), have been shown to exhibit potent antioxidant activity (d' Ischia, 1995; Schmitz et. al., 1995), and therefore protect the melanocytes against harmful free radicals.

1.3 MELANOCYTE BIOLOGY

Mammalian melanocytes are found primarily in the basal layer of the epidermis of the skin, hair follicles, iris and choroid of the eye (Figure 1.2). Epidermal melanocytes extend their numerous dendritic processes between the neighbouring keratinocytes, and it is estimated that about 36 keratinocytes are associated with each melanocyte in this manner. The association between a melanocyte and neighbouring keratinocytes is known as an *epidermal-melanin* unit (Figure 1.2F). Amongst other features, this arrangement ensures that the melanocytic dendrites, which contain melanosomes laden with melanin, are transferred to the adjacent keratinocytes. The colour of skin and hair is related to the number, size, type, distribution and degradation of melanosomes, and to the levels of activity of melanogenic enzymes found within these melanosomes (Bessou et. al., 1996). Further modulation of melanocyte function is brought about by intrinsic and extrinsic factors such as inhibitors, growth factors, hormones and ultraviolet light.

1.3.1 Origin and differentiation of melanocytes

When cells emerge from the neural crest during embryonic development, they enter the "migration staging area", which lies immediately dorsal to the developing somites (see review Newgreen and

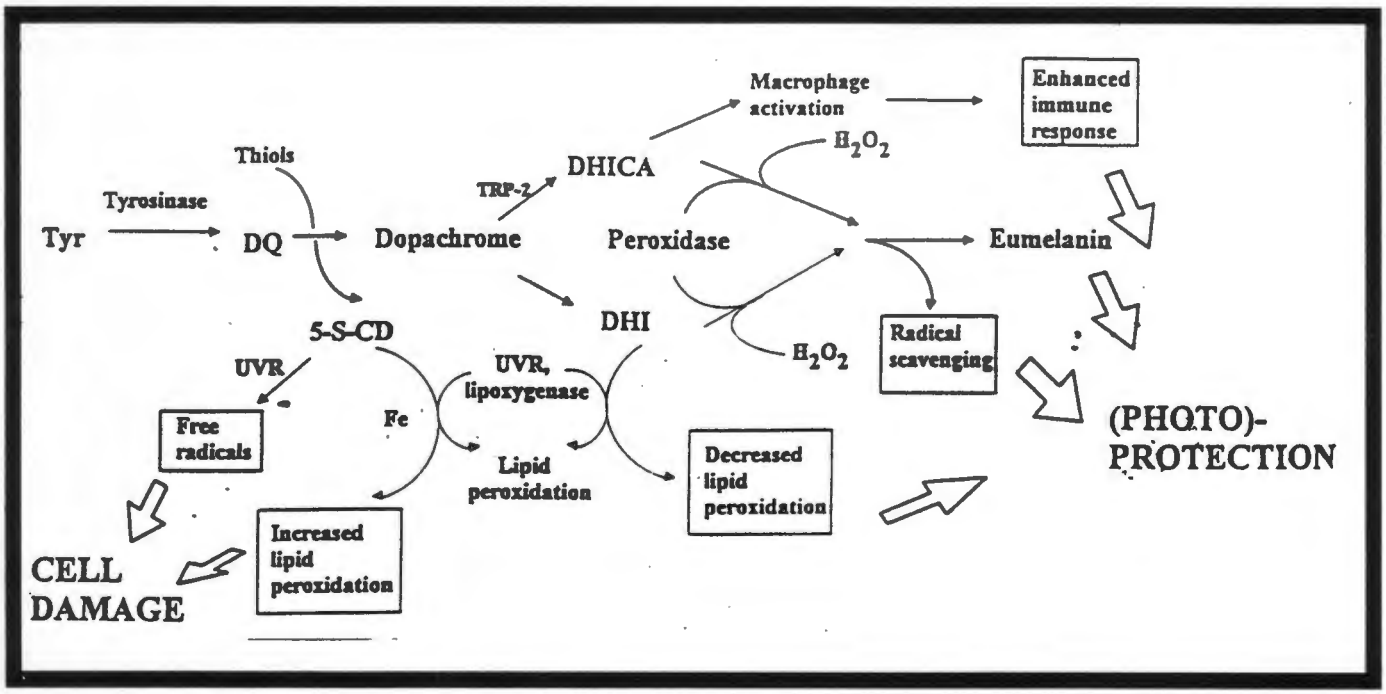


FIGURE 1.1: Schematic outline of Melanogenesis highlighting the role of eumelanogenic intermediates (from Prota, 1997)

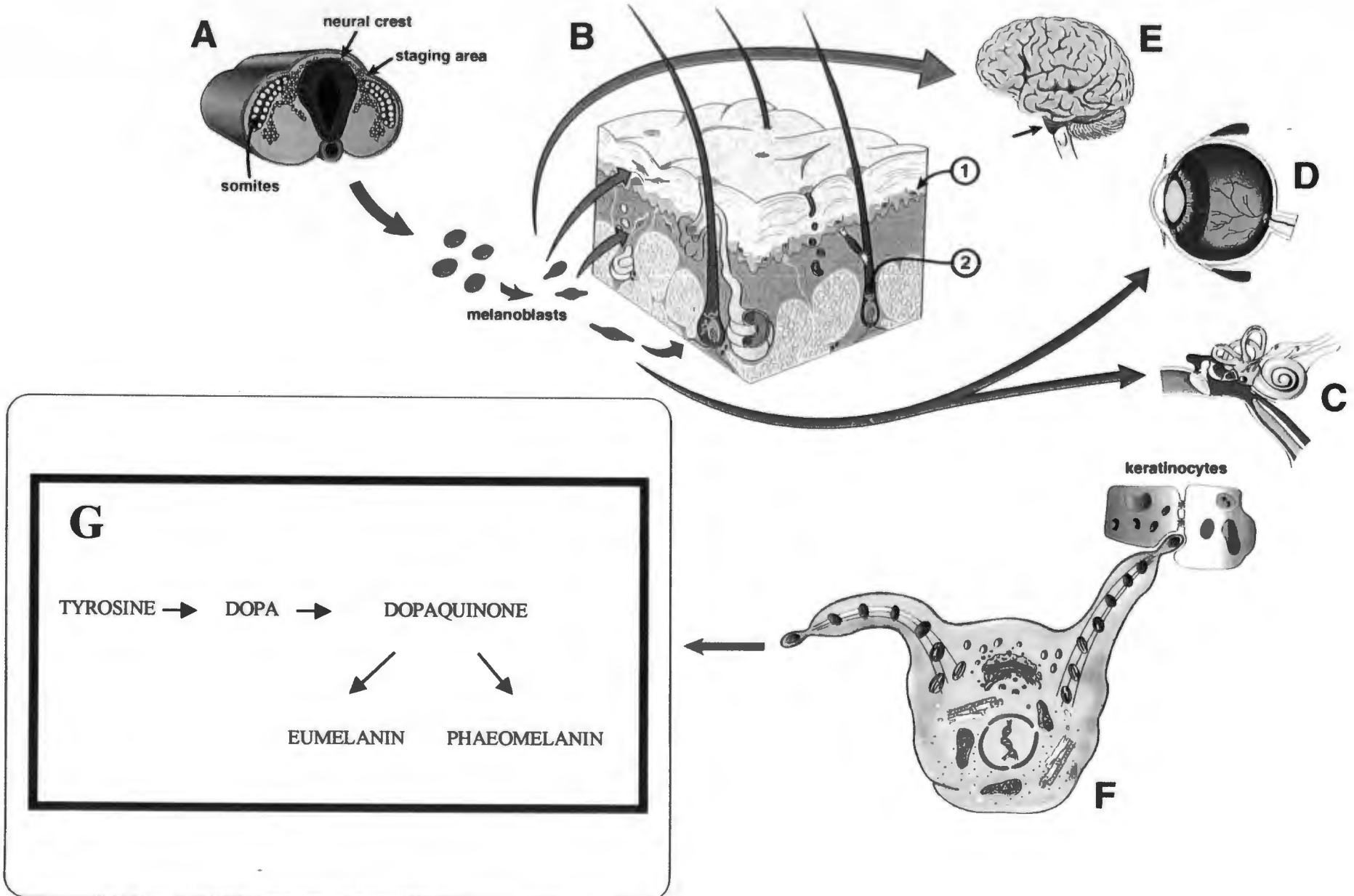


Figure 1.2 : See overleaf

FIGURE 1.2: Overview of melanogenesis. A: Most melanocytes in the body originate embryonically from neural crest cells that emigrate out of the dorsal surface of the neural tube, become committed to melanocytes at the staging area, and migrate selectively along the dorsal/lateral pathway over the somites. B: Melanocyte precursors, melanoblasts, target accurately to specific sites throughout the body. In the skin, these cells eventually reside in the basal epithelial layer of the interfollicular epidermis (1) and in the germinative bulb of the hair-follicle (2). C: In the ears, they eventually reside in the stria vascularis and the modiolus of the cochlea, in the vestibular organ. D: In the eye, they eventually reside in the uveal tract composed of the choroid, ciliary bodies and iris. E: In the brain they reside in the leptomeninges composed of the meninges covering the ventrolateral surfaces of the medulla oblongata and upper cervical cord (see arrows). F: Once settled into these permanent places of residence, the melanocyte completes differentiation and manufactures melanin. A diverse array of melanocyte specific genes are regulated. The corresponding gene products of enzymatic and structural proteins undergo a complete orchestration of translocation in the cytoplasm to form the functional premelanosome. G: In premelanosomes, tyrosine undergoes a series of biochemical alterations to form various melanin polymers. Eventually, in the skin, the melanosomal product is shuttled down the dendrites and transferred to the neighbouring keratinocytes (F). The partnership of an epidermal melanocyte and a neighbouring group of keratinocytes is known as the epidermal-melanin unit. Each epidermal melanocyte secretes melanosomes into a definite number of neighbouring keratinocytes (F). (modified from Boissy and Nordlund, 1997)

Erickson, 1986). From here the neural crest cells, which are destined to become melanocytes, migrate laterally beneath the ectoderm along the dorsolateral pathway. At this stage they are called melanoblasts. Melanoblasts are identified by the presence of TRP-2 (tyrosine-related protein-2), which is the first melanocyte lineage-specific protein to be expressed in melanocytes (Steel et. al., 1992). These melanoblasts migrate throughout the mesenchyme, home into specific target sites and eventually differentiate into active melanocytes, characterised by dendrification (Preston et. al., 1987), detectable tyrosinase activity (Pomerantz, 1966; Pawelek et. al., 1973) and melanin deposition within melanosomes (Hu et. al., 1982).

1.3.2 Summary of melanosome formation

Melanin is synthesized in specialized membrane-bound organelles, the melanosomes, which are located in the melanocyte cytoplasm. The structural components of these melanosomes provide a framework for the activity of the rate-limiting enzyme of melanogenesis, tyrosinase, and for melanin deposition (Jimbow et. al., 1992; reviewed Hirobe, 1992; 1995). The melanosome is formed when Golgi-derived vesicles containing tyrosinase fuses with vesicles or dilated tubules of smooth endoplasmic reticulum that harbour the melanocyte structural components. The fusion of these two membrane compartments initiates the first stage of melanosome maturation. During their maturation, these melanosomes develop into either phaeomelanin (red-yellow pigment)- or eumelanin (black/brown pigment)-producing organelles. Hence, melanosomes in which these are produced are called eumelanosomes and phaeomelanosomes, respectively. Eumelanosomes tend to be ellipsoidal with a regular internal matrix, whereas the spherically-shaped phaeomelanosomes have an amorphous interior. Environmental changes, such as exposure to ultraviolet light, may alter the melanosomal matrix proteins and result in the eventual deposition of melanin within the melanosomes, which are controlled by a number of regulatory melanogenic proteins.

1.3.3 Melanin synthesis

Eumelanins are black to brown and insoluble in most solvents, whereas phaeomelanins are yellow to reddish-brown and alkali-soluble (Ito and Fujita, 1985). At least three melanogenic enzymes are involved in the production of black/brown melanin: tyrosinase, tyrosine-related protein-1 (TRP-1) and tyrosine-related protein-2 (TRP-2). The starting material for the melanogenic pathway is the amino acid, tyrosine (see Figure 1.3). The first two steps of this pathway are common to both eu- and phaoemelanin synthesis, and are catalyzed by the enzyme, tyrosinase. Tyrosinase initiates melanin synthesis by catalysing the hydroxylation of tyrosine to DOPA (3,4-dihydroxy-phenylalanine) and the oxidation of DOPA to DOPAquinone.

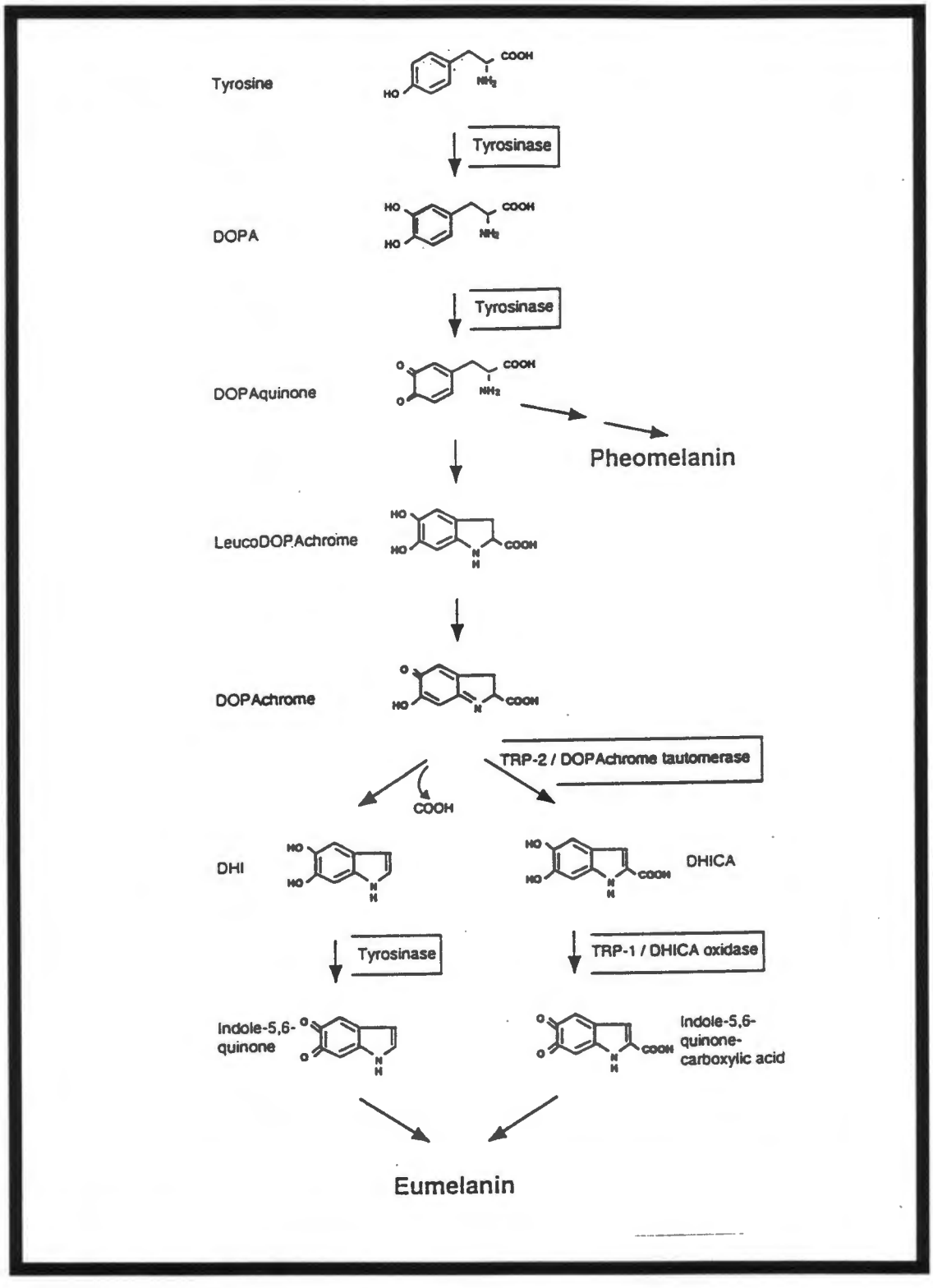


FIGURE 1.3: Schematic diagram of the mammalian melanin biosynthetic pathway, from del Marmol and Beerman, 1996). Starting material is the amino acid, tyrosine, and enzymatic reactions attributed to tyrosinase, TRP-1 and TRP-2.

At this point of the eumelanogenic pathway, the highly reactive intermediate, DOPA-quinone, undergoes a rapid cyclization and rearrangement to sequentially form the indole ring structures leucodopachrome, which is then autocatalytically converted to dopachrome, without the need of a catalytic enzyme. Thereafter, DOPAchrome is converted into 5,6-dihydroxyindole (DHI) either by a spontaneous decarboxylation or enzymatically, under the influence of DOPAchrome tautomerase (DCT) (E.C. 5.3.2.3). DCT catalyses the conversion of DOPAchrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) (Pawelek et. al., 1991). Tyrosinase-related protein-2 has been shown to have DCT activity (Tsukamoto et. al., 1992), and thus their names are used synonymously.

A third enzymatic reaction catalyzed by tyrosinase is the oxidation of 5,6-dihydroxyindole (DHI) to indole-5,6- quinones (Korner and Pawelek, 1982). Furthermore, TRP-1 has been shown to exhibit DHICA oxidase activity, which converts DHICA into 5,6-quinone 2-carboxylic acid (Kobayashi et. al., 1994 a, b). However, in contrast to this result, Boissy et. al. (1997), reported that human TRP-1 does not use DHICA as a substrate for oxidation, but murine TRP-1 does. Finally, the quinonic intermediates from DHI and DHICA conversions form the eumelanin (brown/black) polymers.

1.4 MELANOCYTES OF THE HAIR FOLLICLE

There are two types of hair follicle melanocytes (see Figure 1.4) (reviewed Starrico, 1959; Tobin and Bystryn, 1996; reviewed Castanet and Ortonne, 1997), which differ in their location, size, dendricity and degree of pigmentation. They are: (1) dendritic, pigmented melanocytes located in the hair-bulb, also known as active melanocytes; and (2) non-dendritic and amelanotic melanocytes, located predominantly in the outer root sheath and, to a minor extent in the hair bulb. These amelanotic melanocytes, also known as inactive melanocytes, do not produce melanin and therefore do not react with antibodies to tyrosinase, TRP-1 and TRP-2 melanogenic proteins (Horikawa et. al., 1996).

The function of hair follicle melanocytes is the same as epidermal melanocytes, and that is to produce melanin and provide colour to the hair and skin, respectively. The main difference between the epidermal melanocytes and follicular melanocytes is that the activity and proliferation of the follicular melanocytes are linked with the hair-follicle cycle, whereas the activity of the epidermal melanocytes is regulated mainly by exposure to ultraviolet light. Epidermal melanocytes and hair follicle melanocytes have been shown to be antigenically different (Tobin and Bystryn,

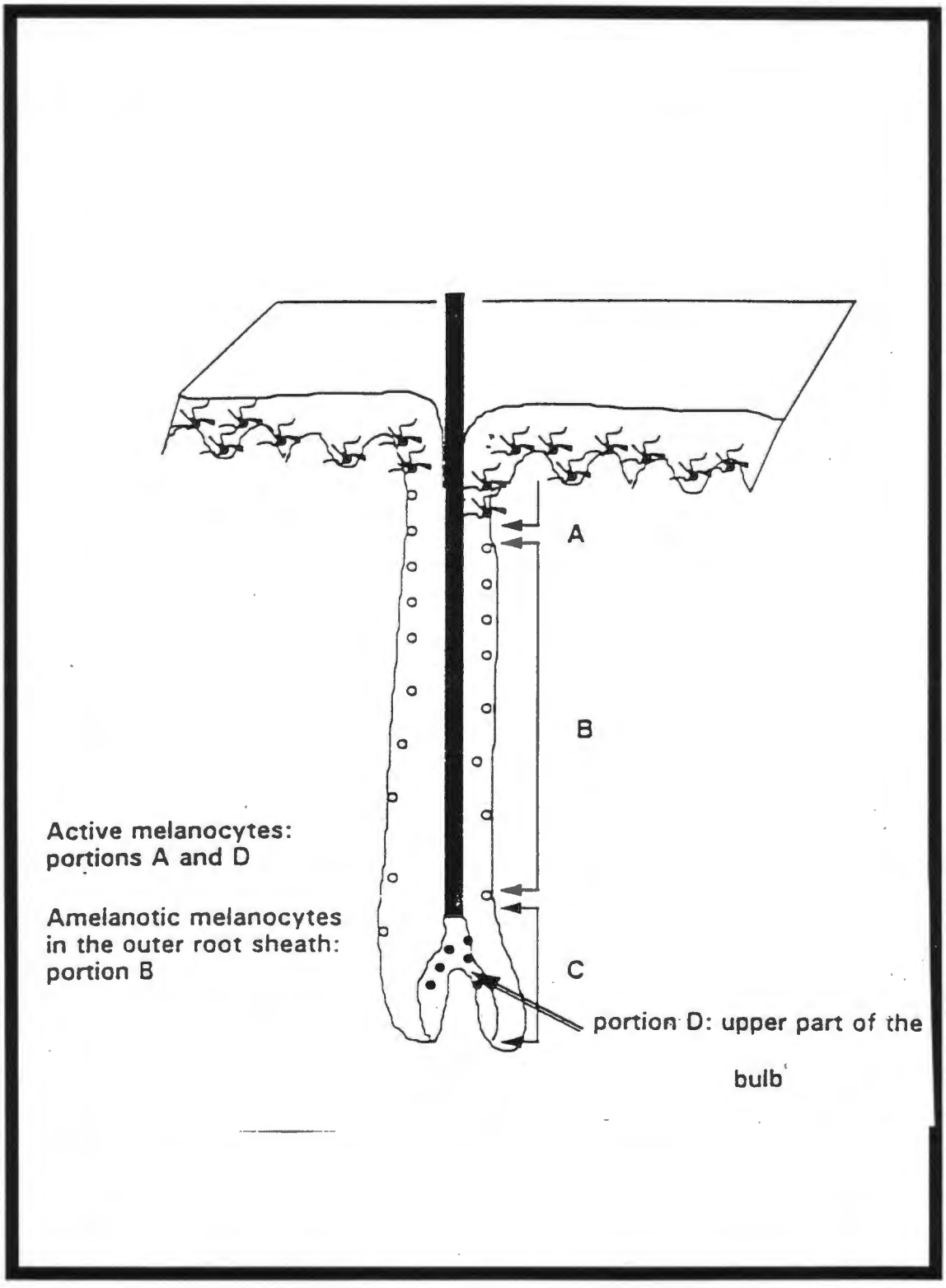


FIGURE 1.4: Subdivision of the hair follicle according to the distribution of melanotic and amelanotic melanocytes. Portions A and D contain melanotic melanocytes while Portions B and C are populated with amelanotic melanocytes (from Castanet and Ortonne, 1997)

1996). This could thus account for the preferential destruction of hair follicle melanocytes in a disease like alopecia areata and of epidermal melanocytes in disease like vitiligo. The colour of hair arises from the transfer of the melanosomes from the hair bulb melanocytes to the keratinocytes in the cortex and medulla of the hair shaft. This only occurs during the anagen (growth) phase of the hair-cycle. This is also the phase in which the enzyme, tyrosinase, is the most active. From the onset of the catagen phase of the hair-cycle, changes occur within the hair follicle resulting in the release of the dermal papilla. Subsequently, the club hair moves towards the skin surface. Above the papilla, the epithelial strand is reduced to the secondary germ. When the next cycle begins, the secondary germ elongates, becomes invaginated by the papilla and a new bulb with melanocytes is formed. These melanocytes come from the reservoir of melanocytes in the outer root sheath, and become differentiated during their migration (Cui et. al., 1991).

1.5 FACTORS AFFECTING MELANOGENESIS

There are numerous endogenous and exogenous factors that may affect skin pigmentation. A brief summary of those factors most concerned with this present study is presented.

1.5.1 Growth factors

1.5.1.1 Keratinocyte-derived growth factors

One of the most important functions of the keratinocytes in skin pigmentation is to release exogenous factors into the epidermal environment after exposure to a stimulus like ultraviolet light. It is well established that factors such as ET-1 (endothelin-1, Yada et. al., 1991; Imokawa et. al., 1992; 1996; 1997), bFGF (basic fibroblast growth factor, Halaban et. al., 1988), and NGF (nerve growth factor, Yaar and Gilchrest, 1991), are released by keratinocytes and are involved in ensuring melanocyte proliferation, migration and differentiation. What is less well known is the role of these factors in inducing the formation of melanocyte dendritic extensions, which serve as the ' vehicle' for transferring of melanin to the adjacent keratinocytes. The role of melanocyte dendricity as a critical factor for skin pigmentation is supported by the report that in a hereditary condition called Griscelli-Pruneiras disease (Hara et. al., 1995), which is characterized by a severe immuno-deficiency, scattered hypopigmented macules and silver-grey hair, the epidermal and follicular melanocytes, contained many mature melanin-laden melanosomes, but possessed short dendritic processes. This suggested that the compromised melanocytic dendrites are responsible for the

hypo-pigmentation, since transfer of the melanin within the mature melanosomes did not take place.

1.5.1.2 *Steel factor:*

Steel factor (*SLF*; also known as *c-kit* ligand, mast cell growth factor or stem cell growth factor) is a growth factor known to play a key role in melanocyte development and differentiation. Mutations of the *steel* gene, or of its cognate receptor (*c-kit* receptor tyrosine kinase) in mice, rats, and humans, result in animals lacking pigments cells *in vivo* (Nishikawa et. al., 1991; Williams et. al., 1992, Dippel et. al., 1995). It is now well established that *SLF* regulates melanocyte development in mice by supporting the survival (Murphy et. al., 1992; Wehrle-Haller and Weston, 1995), development and proliferation of melanoblasts along the dorsolateral pathway (Mackenzie et. al., 1997; Guo et. al., 1997; Kunisada et. al., 1998), before colonizing the skin and then assisting in the differentiation of the melanoblasts into melanocytes (Reid et. al., 1995; Kunisada et. al., 1998). Furthermore, it was suggested that *SLF* played a role in promoting the migration of melanocytes *in vivo*. This was confirmed for the first time in a recent study by Kunisada and co-workers (1998), in which *SLF* was ectopically expressed in keratinocytes and showed that melanocytes were distributed to a number of sites, including the oral epithelium and footpads, where neither melanocytes nor their precursors were usually found.

1.5.2 Melanocyte-stimulating hormone (MSH):

MSH is the best-characterized hormone that is involved in regulation of mammalian pigmentation (reviewed Furumura et. al., 1996 and Luger et. al., 1997). MSH affects both the proliferation of melanocytes (Abdel-Malek et. al., 1992 a, b; 1995; Aroca et. al., 1993; Halaban et. al., 1993; Swope et. al., 1995), as well as stimulating the production of melanins (Burchill and Thody, 1986; Abdel-Malek, 1995). The action of α-MSH on melanocytes *in vivo* and *in vitro* is mediated through one of five closely related G-protein-coupled receptors. All of these receptors are coupled to adenylate cyclase *in vitro*, and most of the melanogenic effects of MSH could be reproduced by treatment with cAMP analogues in a study by (Pawelek, 1985). *In vivo*, α-MSH was shown to increase melanogenesis by the increase in expression of tyrosinase (Levine et. al., 1991; Thody and Graham, 1998). This is possibly by increasing *Mi* (*micropthalmia*) expression, which in turn binds to the M-box of the tyrosinase promoter, thereby leading to the stimulation of tyrosinase expression and melanogenesis. This mechanism is suggested since it was demonstrated in the study of Bertolotto et. al. (1998), that cAMP-elevating agents increased *Mi* transcription and that *Mi* was required for the cAMP effect on the tyrosinase promoter.

1.5.3 Ultraviolet (UV) Light

Ultraviolet (UV) radiation is one of the main external factors that stimulate human melanogenesis. UV radiation comprises three components: UVA (320-400nm), UVB (280-320nm) and UVC (100-280nm). Darkening of human skin after exposure to sunlight (which is mainly UVA light) or UV from artificial sources is commonly known as tanning. This tanning reaction may result from a combination of an immediate pigment darkening (IPD) effect and a delayed pigment darkening (DPD) effect. The IPD effect occurs almost immediately or up to 24 hours after exposure to UVA light, and causes a short-term increase in pigmentation: this pigmentary response results from the photo-oxidation of lightly coloured or colourless melanins to the darker melanin (Willis et. al., 1972). The DPD effect occurs 72 hours after UVB exposure and is accompanied by an increase in melanocyte proliferation and melanogenesis (Rosen et. al., 1987). This increase in melanogenesis results from an up-regulation of the activities of both tyrosinase and TRP-1 enzymes, and an increase in melanocyte dendrification, which ensures an increase in the transfer of melanosomes to the adjacent keratinocytes (Szabo, 1969; reviews Szabo et. al., 1988, and Ortonne, 1990). *In vivo* studies showed that exposure to UVA can also result in increased melanocyte proliferation and melanogenesis (DPD effect), but UVA light brought about similar biological effects to UVB light at UVA doses 1000 times greater than the equivalent UVB dose (Kligman et. al, 1985, Rosen et. al., 1987).

Little is known of the molecular mechanisms underlying the IPD and DPD effects. Several pathways and factors have been implicated in increasing melanogenesis and/or proliferation of melanocytes. The most studied consequence of UV exposure, is the enhanced melanogenic process. The first pathway implicated for the UV-induced melanogenesis is the diacylglycerols / protein kinase C pathway (DAGs / PKC) (Gordon and Gilchrest, 1989; Punnonen and Yuspa, 1992). UVB was shown to interact with the membrane lipids and this resulted in the release of diacylglycerols (DAGs, a phospholipid derived 1,2-diacylglycerol) from the membranes (Carsberg et. al., 1995; Friedman et. al., 1990). This theory was supported by a study in which DAGs were applied to guinea pig skin resulting in a pigmentary response similar to that seen after UV-induced tanning (Allan et. al., 1995). DAGs enhanced melanin formation by increasing PKC-mediated phosphorylation of tyrosinase, resulting in an increase in melanin formation (Park et. al., 1993 a, b).

A second possible pathway for UV-induced melanogenesis was suggested to be via the cGMP pathway. This process resulted from the release of nitrous oxide (NO), a paracrine factor released from the adjacent keratinocytes after the exposure to both UVA and UVB irradiation's *in vitro*

(Romero-Graillet, 1997), and was reported to be responsible for the UVB-induced sunburn reaction *in vivo* (Warren et. al., 1994). It was proposed that NO increased melanogenesis by mimicking the melanogenic effects of UVB. This was confirmed in studies by Romero-Grailett et. al. (1996; 1997), who demonstrated that NO donors increased the activities of both tyrosinase and TRP-1 enzymes, and consequently melanin synthesis in melanocytes. In contrast, the melanogenic stimulatory effects were almost completely reversed in the presence of NO scavengers. In the same study, it was shown that exposure to UVB light caused a marked increase in the cGMP content in these melanocytes. It was also shown that inhibitors of NO synthase (the enzyme which synthesizes NO), guanylate cyclase and cGMP-dependent protein kinase (PKG) blocked the melanogenic effects of UVB. This possibly demonstrated that the NO-cGMP pathway, through activation of PKG, was involved in UVB-induced melanogenesis.

Another mechanism for UV-induced melanogenesis and melanocyte proliferation could be explained by the direct action of paracrine factors on melanocytes. The paracrine factor, endothelin-1 (ET-1), is known to increase both melanocytic mitogenesis and melanogenesis (Imokawa et. al., 1992; 1993; 1996; 1997). The increase in melanogenesis was shown to be due to the up-regulation of the activities and mRNA synthesis of both tyrosinase and TRP-1. An *in vitro* study by Imokawa et. al. (1997) demonstrated that these biological effects resulted from cross talk between the PKC, PKA and MAP kinase signal transduction pathways which was induced by ET-1 (see Figure 1.5). Other paracrine factors, in addition, have also been shown to directly increase the pigmentary response of the skin. For example, nerve growth factor (NGF) released from UV-induced keratinocytes was shown to be responsible for ensuring the survival of the melanocytes after UV exposure. This was due to increased levels of BCL-2 protein, which prevented apoptosis of cells (Zhai et. al., 1996).

Lastly, a mechanism by which UV light might induce melanogenesis and proliferation has been linked to DNA damage and/or repair mechanisms (Eller et. al., 1994; 1996, Pedeaux et. al., 1998). Exposure of cells to both UVA and UVB resulted in DNA damage, either by the release of ROS (reactive oxygen species), causing oxidative DNA damage, or by the formation of DNA photo-adducts between the pyrimidine bases of the DNA (reviewed Applegate and Frenk, 1995; and Gilchrest, 1996). This damage was recognized and repaired by a nucleotide excision repair process (Tornaletti and Pfeiffer, 1996). The repair mechanisms involved the removal of the pyrimidine dimers and photoproducts induced by UVA and UVB, respectively. During this repair process, short DNA fragments containing the dimers or photoproducts were released, which restored the

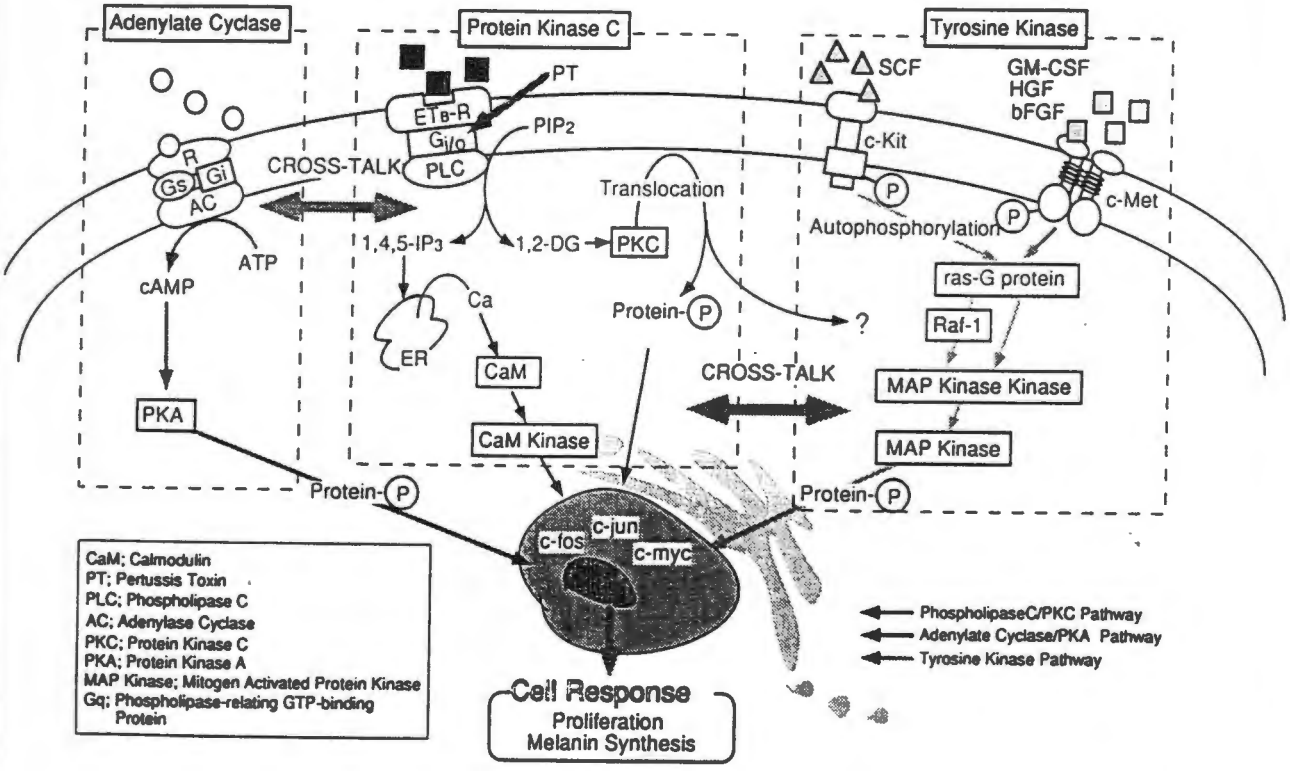


Figure 1.5: Schematic diagram of intracellular signal transduction systems in endothelin-1 (ET-1)-stimulated melanocyte proliferation and melanogenesis of human melanocytes (from Imokawa et. al., 1997)

integrity of the DNA. Investigators have demonstrated in cell culture experiments that the addition of DNA-damaging chemicals or small DNA fragments or pyrimidine dinucleotides (pTpT), which mimicked the fragments released during the excision/repair process, increased the melanin content of these cells (Eller et. al., 1994, 1996; Pedeaux et. al., 1998). It was further shown that the DNA-damaging chemicals and pTpT fragments increased the expression of MSH receptors and MSH binding in both melanoma cells and normal human melanocytes. Thus, these DNA repair fragments were likely to be responsible for the observed increase in proliferation and melanogenesis after UV exposure, because MSH was known to increase both melanocyte proliferation and melanogenesis *in vivo* (reviewed Luger et. al., 1997).

Several other factors are thought to play a role in the biosynthesis of melanin, but their specific functions have not yet been clarified. These factors include, for example, endogenous melanogenic inhibitors (Hatta et. al., 1988; Kameyama et. al., 1989), catalases (Halaban and Moelmann, 1990), peroxidases (d'Ischia et. al., 1991, Gesualdo et. al., 1997), and *agouti* genes (Wilson et. al., 1995).

1.6 VITILIGO

Vitiligo is a depigmenting disease that affects persons of all racial groups and both sexes. It usually spreads from the extremities of the body, but may also commence on any part of the integument. The characteristic irregular milk-white spots on the exposed areas of the body tend to enlarge centrifugally over time. For reasons as yet unknown, lesions may spontaneously repigment in some patients. A trichrome pattern of colouration is relatively common in dark-complexioned patients, with central depigmentation, peripheral hyperpigmentation and surrounding skin of normal pigmentation, thus making lesions more visibly pronounced in these darker-complexioned patients (see Hann et. al., 1997, for a recent review on clinical features of vitiligo).

1.6.1 HISTOLOGICAL CHANGES IN VITILIGO MACULES

The question of whether lesional melanocytes die (or merely turn-off melanogenesis) is central in understanding the treatment of vitiligo. Several investigators have addressed this question (reviewed Le Poole and Das, 1997). The extensive immunohistochemical studies by Le Poole et. al. (1992; 1993; 1996) and Norris et. al. (1996) are most convincing, as they include antibodies to

detect melanocytes based on both pigmentary and other melanocyte-specific phenotypic characteristics. (This was unlike the approach used previously by other investigators, who mainly used antibodies that were only able to detect melanocytes actively involved in melanogenesis). The overall conclusions from the studies by Le Poole et. al. (1993, 1996), are that melanocytes are completely absent in long-standing / stable lesions (lesions which had not progressed in size for more than a year). Melanocytes, however, were found to be present in active / progressing lesions, although fewer in number and seeming to have undergone degenerative changes compared with the melanocytes found in non-lesional skin. With the use of a confocal laser-scanning microscope, these degenerated melanocytes appeared to be grossly enlarged, bizarre-shaped, fragmented and displayed retracted dendrites. The question of whether hair-follicle melanocytes were affected at all in vitiligo patients was not addressed in any of these investigations. However, it has been mentioned in clinical reports of vitiligo patients, that the hairs found in lesional skin are usually pigmented, suggesting that these melanocytes of the hair follicle were somehow protected from this disease.

In the study by Norris et. al. (1996), *c-kit* antibodies were used to detect melanocytes in punch biopsies from lesional, peri-lesional, non-lesional and normal skin. The findings from his study confirmed that *c-kit* protein was present on all adult melanocytes, and that the number of *c-kit* positive melanocytes in non-lesional skin was the same in normal, human control skin samples. Furthermore, it was shown that the melanocytes were absent in lesional skin and decreased in number in peri-lesional, compared to non-lesional biopsies and normal human skin biopsies. These *c-kit* results agreed with those of Le Poole et al. (1993), since staining with *c-kit* was only observed in peri-lesional vitiliginous skin and adjacent normal skin, but absent in lesional vitiliginous skin.

In addition to the changes of the melanocytes, other cells of the vitiliginous epidermis were also affected. Moelmann et. al. (1982) and Bhawan et. al. (1983), demonstrated that vacuolar damage to the basal keratinocytes occurred in vitiligo lesions. This damage to the keratinocytes was focal and most extensive in areas immediately adjacent to melanocytes, which suggested that the injury to the keratinocytes was secondary to the release of toxic material from damaged melanocytes. On the other hand, damage to keratinocytes could have been caused by another factor, and these damaged keratinocytes could then have affected the survival of the adjacent epidermal melanocytes. In addition, Hann et. al. (1992), suggested that a leukocyte infiltrate, found in the epidermis of spreading vitiligo lesions in his study, was associated with the vacuolar changes in the basal keratinocytes and melanocytes. It was therefore suggested that the damage to these epidermal cells

was caused by the local milieu, which eventually led to the damage and ultimately, the loss of these melanocytes in the lesions.

Inflammatory cells were also found to be present in vitiligo lesions. Lesions from patients with inflammatory vitiligo, which was recognized by the border of the depigmented macule being red and frequently itchy, had a higher content of inflammatory cells than that found in generalized vitiligo lesions (Abdel-Naser et. al., 1994; Le Poole et. al., 1996). The composition of the cell infiltrate consisted mainly of T lymphocytes (Le Poole et. al., 1996), which were more numerous in peri-lesional skin, than in the control skin. In addition, they were mainly concentrated where melanocyte destruction took place and where macrophages were found (Badri et. al., 1993). Langerhans cells, which are one of the most important cells involved with the immunity of the skin (Misery et. al., 1997; 1998), were also increased in number in peri-lesional vitiligo skin, when compared with that in non-lesional and lesional skin. An alteration in the number of inflammatory cells in peri-lesional vitiligo skin, compared to the control skin, suggested that a local immune reaction could be involved in the degenerative process of melanocytes in vitiligo.

1.6.3 CAUSES OF VITILIGO

The factors responsible for causing vitiligo should ideally be known so that they could be removed or inhibited during treatment. The exact aetiology of vitiligo remains unknown. This could therefore account for the variable results obtained with different treatment modalities. The actual cause of vitiligo is one of the most debated questions around this disease, and unfortunately, there is no plausible answer as yet. From previous studies it would seem that the mechanisms of melanocyte destruction are likely to be a combination of many different processes influencing melanocyte function and survival. Several theories have been proposed for the pathogenesis of vitiligo, but because it is beyond the scope of this study, a brief summary of the most prominent theories are described below.

The **neural theory** proposes that a neural dysfunction exist in vitiligo patients. Ultrastructural studies of the dermal nerves from biopsies taken from marginal and central vitiliginous skin lesions, showed mild degenerative changes in a small proportion of the axons and Schwann cells of the nerves which supplied these lesions (Al' Abdie et. al., 1995). Since a communication pathway between the nervous system and epidermal melanocytes exists (Hara et. al., 1996), an alteration in

the structure or function of the nerves would disturb the function of the cells they supplied. An alteration of nerves would possibly result in abnormal levels of neurotransmitters being released from the nerve-endings, which could be toxic to the melanocytes (reviewed Ortonne and Bose, 1993). The reported abnormal levels of both catecholamines (Morrone et. al., 1992; Chakraborty and Chakraborty, 1996) and acetylcholine (Elwary et. al., 1997) neurotransmitters in vitiligo patients, strengthens this proposal.

The **self-destruct** theory proposes that vitiligo results from the destruction of melanocytes by toxic products made by the melanocytes themselves (reviewed Ortonne and Bose, 1993; Pawelek et. al., 1980). There are several variants of this hypothesis. Firstly- there is an overproduction of or an inability to inactivate certain enzymes and /or intermediate products of melanin synthesis, which are regarded to be toxic to the melanocytes, for example, hydrogen peroxide. Secondly- there are multiple defects in catalase (an enzyme that inactivates hydrogen peroxide) activity (Maresca et. al., 1997; Passi et. al., 1998). This enzyme is also involved in catecholamine synthesis in the epidermis of vitiligo patients, and therefore the abnormal catecholamine metabolites mentioned previously, could be explained (Morrone et. al., 1992; Schallreuter et. al., 1996). Defective catalase activity leads to an accumulation of hydrogen peroxide in the epidermis, which is damaging to the melanocytes and surrounding cells. Thirdly- melanin fails to remove toxic free radicals, the accumulation of which kills the cells.

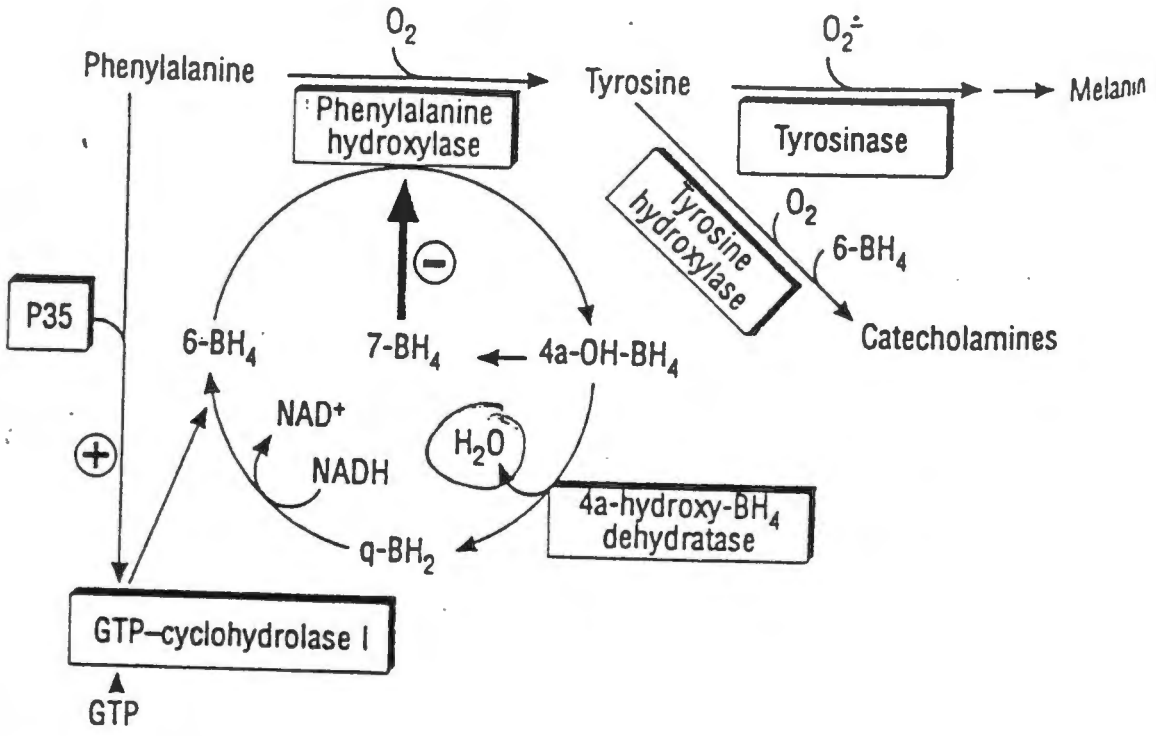
A number of observations, supporting the **autoimmune theory**, include the following:
(1) The most convincing evidence that vitiligo is an autoimmune disease, was the demonstration that there were specific antibodies to melanocyte cell-surface antigens present in the blood circulation of most vitiligo patients. These antibodies were initially demonstrated by immunoprecipitation (Naughton et. al., 1983; Mitchell et. al., 1980) of melanocyte-surface antigens and by indirect immunofluorescence (Bystryn et. al., 1985). Their presence was confirmed by other investigators using additional techniques such as complement-dependent cytotoxicity (Norris et. al., 1988), antibody-dependent cellular cytotoxicity (Yu et. al., 1993; Norris et. al., 1988), immunoblotting and by live-cell enzyme-linked immunoabsorbent assay (ELISA) and passive transfer experiments (Harning et. al., 1991; Gilhar et. al., 1995, Kemp et. al., 1996; 1997 a,b; 1998; 1999; Baharav et. al., 1996). Furthermore it was reported that these antibodies correlated with the extent and activity of the disease, and have the ability to kill melanocytes *in vitro* and *in vivo* (Harning et. al., 1991; Gilhar et. al., 1995, Durham-Pierre, 1995). Moreover, it was shown that the titre of melanocyte antibodies decreased in vitiligo patients that responded to PUVA therapy,

confirming that the antibody titre was related to the disease activity (Hann et. al., 1997).
(2) Numerous patients with vitiligo have other autoimmune diseases including diabetes mellitus and alopecia areata (Lerner et. al., 1978). In addition, a large proportion of patients with vitiligo showed a 2-10 fold increase in the auto-antibodies to numerous organs, particularly the adrenals and thyroid (Gokhale et. al., 1983; Mandry et. al., 1996).
(3) Relationships between vitiligo and melanoma suggested that immune reactions targeting melanoma cells could also destroy normal human melanocytes (Cui and Bystryn, 1995). This was supported by the clinical observation that melanoma was often associated with a vitiligo-like depigmentation within, around, or distal to the melanoma (Bystryn et. al., 1987; Merimsky et. al., 1994; 1996). The up-regulation of Interleukin-2 (IL-2) was also reported to cause vitiligo in patients with melanoma, but not in those with other diseases (Richards et. al., 1992). It was presumed that in these patients, IL-2 up-regulated preexisting immune-cell responses to pigment cell antigens on melanoma, resulting in the destruction of normal human melanocytes in the process. Support for the up-regulation of IL-2 in vitiligo patients is provided in the recent study of Yeo et. al., 1999.

The **oxidative stress theory** proposes that melanocyte destruction result from the depletion of anti-oxidants or the increase in the levels of reactive oxygen species (ROS) and other free radicals within the melanocyte. This results in the anti-oxidant levels becoming insufficient to counteract these radicals. This was supported by studies that showed that both non-enzymatic anti-oxidants, including vitamin E, GSH (glutathione), total ubiquinone ($CoQ_{10}H_2$ and CoQ_{10}) and the enzymatic antioxidant, catalase (CAT), were significantly reduced in vitiligo patients compared to the levels in the control patients (Schallreuter et. al., 1994 a, b; Maresca et. al., 1997; Passi et. al., 1998). Further support for the destruction of melanocytes by free radicals was shown in a study by Passi et. al. (1998), who demonstrated that a reduction in phospholipid-polyunsaturated fatty acids occurred in vitiligo patients, compared to normal patients. When there is an increase in ROS in a cell, lipoperoxidation of cell membranes takes place, and this involves the use of phospholipid fatty acids. Therefore the higher the levels of ROS in the epidermis, the lower the levels of phospholipid fatty acids in the epidermis (reviewed Applegate and Frenk, 1995). Thus, reduced levels of phospholipid-polyunsaturated fatty acids of vitiligo patients could be explained possibly by the increasing levels of free radicals within the epidermis of these patients. These studies thus support the oxidative-stress theory that suggests that the free radicals that cause damage to the melanocytes are the initial pathogenic event, which triggers melanocyte degeneration in vitiligo.

The oxidative stress theory also ties in with another theory for melanocyte destruction, which proposes that **defective recycling of tetrahydrobiopterins** in the epidermis of vitiligo patients, results in the depigmentation of skin lesions. This theory is briefly explained in conjunction with Figure 1.6. At this stage, in my opinion, this theory appears to be the most plausible for explaining the pathogenesis of vitiligo, since it attempts to account for most of the biochemical changes observed in vitiligo patients. Thus, all the theories mentioned above, except for the autoimmune theory, could possibly co-exist. This theory is based on the fact that in vitiligo patients, there is a decrease or an absence of the enzyme, 4a-hydroxy-BH_4 [-5,6,7,8-tetrahydrobiopterin] dehydratase (Schallreuter et. al., 1994 a, b, c). To explain how the auto-immune theory can co-exist with the other theories, it is in my opinion that the initial decrease of the enzyme 4a-hydroxy-BH_4 [-5,6,7,8-tetrahydrobiopterin] dehydratase results from an immune reaction which targets this pathway and eventually leads to an interference in the synthesis of this enzyme. Since the states of immunity for the various individuals will be different, the degree of interference of enzyme synthesis will also vary. Consequently, the degree of depigmentation and also the biochemical changes that are observed in the different individuals will vary. To test this theory, a study should be done to determine whether the patients who spontaneously repigmented, have had a boost in their immune systems. This can be done by comparing their T cell counts found within the depigmented areas to those counts found within in the area that spontaneously repigmented. Furthermore, a study should be carried out to compare the 7-BH_4 levels of vitiligo patients, who are repigmenting (spontaneously or after treatment), to the levels before they had started repigmenting, in order to shed some light on the feasibility of this theory.

An explanation on how the decrease in the levels of 4a-hydroxy-BH_4 dehydratase could possibly result in the skin depigmentation will now follow. The decrease in this enzyme may result in an accumulation of 7- BH_4 (7-tetrahydrobiopterin) in the epidermis, which is an inhibitor of the enzyme, phenylalanine hydroxylase (PAH), which is responsible for the conversion of phenylalanine to tyrosine. Thus, inhibition of PAH activity, means less phenylalanine will be converted into tyrosine. Consequently, depigmentation will occur, since tyrosine is the starting material for pigment synthesis. The decrease in PAH activity in vitiligo patients was confirmed by the abnormally high phenylalanine levels seen in the *in vivo* study by Schallreuter et. al. (1998). Furthermore, the accumulation of 7-BH_4 and phenylalanine, see Figure 1.6, may lead to an increase in the levels of hydrogen peroxide. This may possibly result in the altered catalase activity in vitiligo melanocytes, as demonstrated by several investigators (Schallreuter et. al., 1991; Maresca et. al., 1997). In addition, the accumulation of phenylalanine is said to set up a feedback



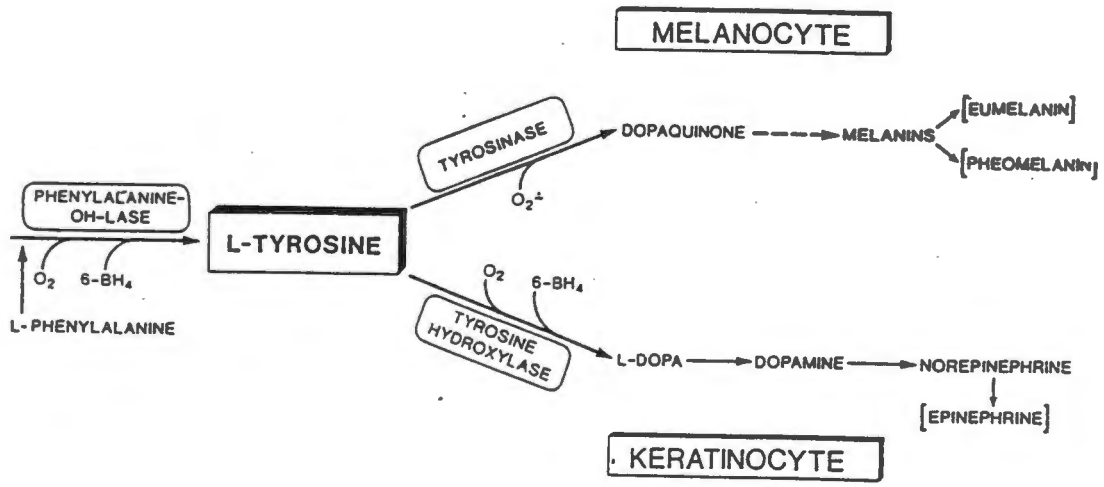


FIGURE 1.6 Above: Schematic diagram of the *de novo* biosynthesis and recycling of 6-BH_4 in regulation of L-tyrosine production by phenylalanine hydroxylase in the human epidermis (from Schallreuter et al., 1994a)
Below: Schematic diagram indicating that L-tyrosine is the common substrate for melanin biosynthesis by melanocytes and catecholamines biosynthesis by keratinocytes (from Schallreuter et. al., 1994b)

mechanism which leads to increased levels of the biopterins (6- BH_4), which are converted into 7-BH_4, and thus may lead to even further depigmentation. These biopterins were also shown to be toxic to melanocytes *in vitro* (Schallreuter et. al., 1994 a, b, c). Because of the ability of these biopterins to fluoresce after exposure to UVA light, it is clinically possible to differentially diagnose vitiligo from other leucodermas. The increase in 6-BH_4 levels may also result in an increase in catecholamines synthesis in keratinocytes (see Figure 1.6), which in turn could explain the increased monoamine oxidase activity observed in vitiligo patients (Schallreuter et. al., 1996). Moreover, this theory also provides an explanation for the increased plasma and urinary levels of catecholamine metabolites observed in vitiligo patients (Moronne et. al., 1992; Schallreuter et. al., 1994c).

1.7 VITILIGO THERAPY

A large variety of therapeutic agents are being or have been tested for the treatment of vitiligo (reviewed Grimes et. al., 1993; Nordlund and Majumder et. al., 1997; Falabella et. al., 1997; Mandel et. al., 1997). PUVA photochemotherapy, which involves combining the drug psoralen (a furocoumarin) with UVA, is the treatment that currently provides the most satisfactory results for repigmenting the skin of vitiligo patients. PUVA photochemotherapy has been shown to increase the number of epidermal DOPA (L-3-4-dihydroxy-phenylalanine)-positive melanocytes, resulting in an increase in melanin production *in vivo* (Blog and Szabo, 1979; Park et. al., 1990). The exact mechanism by which PUVA brings about its biological effects is not known, nor is its pharmacological action entirely clear.

The first clues as to how PUVA acts were deduced from the observation that peri-follicular spots, which were "islands" of pigment around the hair follicles, arose when patients responded to PUVA therapy. These spots eventually coalesced and formed larger areas of pigmentation. In contrast, vitiliginous skin in the non-hair bearing body regions did not repigment at all or repigmented very little after PUVA treatment. This suggested that PUVA treatment stimulated pigmentation of the depigmented skin by stimulating the migration of the melanocytes from the hair follicle. Light microscopic and ultrastructural studies supported this theory by demonstrating that PUVA enhanced the repigmentation of vitiliginous skin by stimulating the inactive melanocytes in the outer root sheath (ORS) of the hair follicle to divide, to proliferate and to migrate upward along the surface of the ORS, into the nearby depigmented epidermis (Ortonne et. al., 1979; 1980; Cui et. al., 1991). In

addition, the melanocytes at the borders of vitiliginous lesions were also shown to be stimulated in this manner.

In order to understand the mechanism of this peri-follicular spreading, it was proposed that the highly proliferative melanocytes within the ORS, which acted as a reservoir, were preferentially stimulated by the growth factors released from the PUVA-irradiated epidermal cells. To test this theory, Abdel-Nasser et. al. (1997) grew melanocytes in the sera of patients that had been irradiated with PUVA for several months, and as a control, grew melanocytes in the sera drawn from the same patient prior to PUVA irradiation. The proliferative rate of the melanocytes grown in PUVA-irradiated sera tripled that of the melanocytes grown in non-PUVA irradiated sera. It was therefore proposed that PUVA acted on epidermal keratinocytes or dermal components to release melanocyte growth stimulatory factors into the sera of these patients, thus enhancing the proliferation and later the migration of melanocytes into the depigmented epidermis.

A second proposed theory for the mechanism of action of PUVA was based on the fact that DNA damage enhanced melanogenesis and melanocyte proliferation, as discussed previously under *ultraviolet light*. Both, psoralen alone and the photochemical product of the combination of psoralen and UVA, damage DNA by intercalation with DNA bases, formation of DNA photoadducts or by the formation of reactive oxygen species (Morliere et. al., 1988; Kinley et. al., 1994; Gasparro et. al., 1997). This damage would result in the release of fragments during the repair process of the cells, which possibly enhances MSH activity (Pedeaux et. al., 1998; Gasparro et. al., 1997). Consequently, there is an increase in the content of photo-protective eumelanins within the cells and also an increase in melanocyte numbers *in vivo*, as explained previously.

A third theory proposed for the increase in melanocyte number and melanogenensis by PUVA also involves the action of MSH. Both PUVA and UVB were shown to enhance the number of epidermal DOPA-positive melanocytes and melanin production *in vivo* (Szabo et. al., 1969; Ortonne et. al., 1979; 1980; Blog et. al., 1979; Kinley et. al., 1994; Gilchrest et. al., 1996). *In vitro*, however, PUVA and UVB inhibited melanocyte proliferation but enhanced melanin production (Kao and Yu, 1992; Abdel-Malek et. al., 1994; Mengeaud and Ortonne, 1996). An explanation for the growth inhibitory effects with PUVA and UVB is possibly explained by the increase in the G2 phase of their cell cycle of cultured melanocytes exposed to UVB light (Abdel-Malek et. al., 1994). This is the phase during which repair of the cell takes place and the cell "protective" genes, for example, p53 tumour-suppresser genes, may be up-regulated (Im et. al., 1998). However, *in vivo*,

this is the phase in which MSH activity is the highest, and melanocytes will probably become more responsive to MSH. Therefore, prolonging this phase possibly may result in pre-existing melanocytes to generate new functional melanocytes (increased proliferation) and an increase in melanin production may also occur. It can thus be predicted that if long-term cultures of PUVA-treated melanocytes were carried out (a study not yet done by any investigator), the initial inhibition of melanocyte proliferation would probably be followed by an increase in melanocyte proliferation, once the initial damages by PUVA and UVB were overcome.

The fourth proposed theory for PUVA-induced melanogenesis, is the formation of psoralen-fatty acid adducts, which stimulate melanogenesis via the PKC signal transduction pathway. Investigators reported that PUVA photo-products intercalated with the phospholipids of the cell membranes and formed psoralen-fatty acid adducts (reviewed Zarebska et. al., 1994). These psoralen-fatty acid adducts were recently isolated by the investigators, Anthony et. al. (1997) and Frank et. al. (1998), and it was demonstrated that they were similar in structure to diacylglycerols (Caffieri et. al., 1996). Since DAGs are known to increase tyrosinase activity via the PKC pathway (Gordon and Gilchrest, 1989; Farago and Nishizuka, 1990; Park et. al., 1993), it was suggested that psoralen-fatty acid adducts increased melanogenesis by mimicking DAGs and thus stimulated this pathway. This theory was supported in part, by the study of Frank et. al. (1994), who demonstrated that psoralen-fatty acid adducts increased tyrosinase activity and melanin production in cultured human melanocytes, whereas the fatty acids alone and the psoralen itself had no effect on melanogenesis. Furthermore, a recent investigation by Anthony et. al. (1997), showed that when 8-MOP (8-methoxypsoralen)-fatty acid adducts were substituted for DAGs in a cell free system, there was an increase in the PKC pathway. Finally, the tumour promoting phorbol esters were reported to have a DAG-like structure (Castagna et. al., 1982; Nishizuka, 1984; 1988), and it was suggested that PUVA mimicked the action of these tumour promoters and caused an increase in melanocyte proliferation and melanogenesis. If PUVA carried out its mechanism along the same pathway whereby these phorbol esters carried out their function, both the repigmentary and carcinogenic reports of PUVA treatment could therefore be accounted for.

In the last theory for PUVA-induced melanogenesis, the melanocytes are indirectly affected. This theory proposes that PUVA have an immunosuppressive effect on the skin, which creates a favorable milieu for melanocyte survival and growth. The immunosuppressive effects of PUVA were postulated because several investigators showed that psoralen, 8-MOP, PUVA and UVA decreased the proliferative rate of cells involved in the immunity of the body. For example, the rate

of proliferation of human T lymphocytes was demonstrated to be decreased *in vitro* (Badri et. al., 1993). In addition, a decrease in $CD4^+$, $CD8^+$, and IL-2 receptor expressions were also reported in PUVA-treated vitiligo patients *in vivo* (Johnson et. al., 1996; Lauharantu, 1997). The decrease in antibody titres against melanocytes observed after PUVA therapy of vitiliginous skin also supported the immunosuppressive role of PUVA (Hann et. al., 1997).

1.8 REASONS FOR THE SEARCH FOR AN ALTERNATIVE TREATMENT FOR VITILIGO

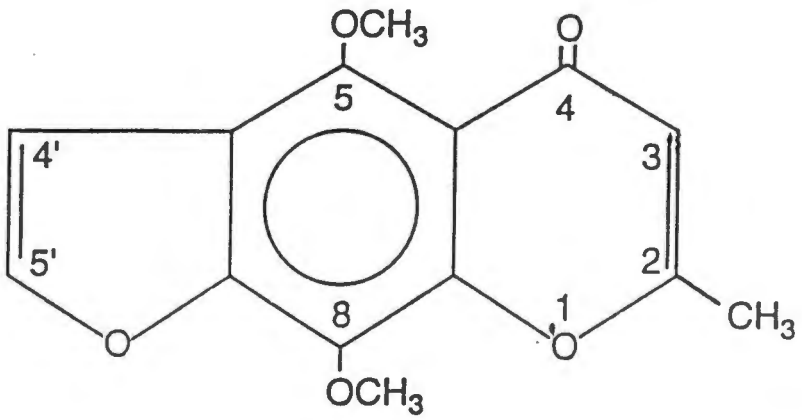
There are numerous unwanted adverse effects of PUVA treatment (Wolff, 1990; Weinstock et. al., 1995; Leroy et. al., 1996; Buckley and Rogers, 1996; reviewed Lauharanta et. al., 1997; Wang et. al., 1997). These can be divided into short and long-term adverse effects. The short-term and most common adverse effects include a phototoxic, erythematous skin reaction, pruritus, nausea and headaches. While the short-term adverse effects are limited and reversible after discontinuation of treatment, potential long-term adverse effects such as accelerated skin aging, dyskeratotic skin conditions, melanoma and non-melanoma skin cancer, immunological alterations and cataract formation are of great concern. Children under 12 years of age have a greater risk of developing cataracts and irreversible retinal damage than older patients, since they have not yet developed protective chromophobes. During a typical photochemotherapy session, the patient is exposed to photons of ultraviolet-A light, and the chromophobes are responsible for the absorption of excessive photons. Thus, damage to the retina occurs if these chromophobes are decreased in number. The increased risk of carcinogenesis with PUVA photochemotherapy is possibly explained by the fact that the newly formed photochemical psoralen derivative, which intercalates with cellular DNA, results in errors of the DNA repair process. In addition, the immunosuppressive side effects of PUVA may also partly explain the tumour-promoting effect (as suggested above) of PUVA, since the immune system is important for recognising and eliminating transformed cells. In addition, of great concern to the light-complexioned vitiligo patient, is the fact that PUVA could repigment adjacent normal skin as well. Therefore the contrast in colour between the normal and vitiliginous skin, is still visible. An alternative treatment for vitiligo is thus being sought.

1.9 KHELLIN AND UVA (KUVA), AS AN ALTERNATIVE TREATMENT FOR VITILIGO

Khellin is a naturally occurring furochromone, which is isolated from the seeds of *Ammi visnaga* (Arabic khella), and is similar in structure to psoralens (Figure 1.7). When khellin was combined with UVA, it was reported to repigment vitiligo skin as effectively as PUVA photochemotherapy (Abdel-Fattah et. al., 1982; Honigsman et. al., 1985; Ortel et. al., 1988; Orrechia and Perfetti, 1992), and without the adverse effects reported with PUVA treatment (Wolff, 1990). In contrast to PUVA, the occurrence of a phototoxic skin reaction with KUVA photochemotherapy, was unreported. In addition, KUVA formed a lower number of crosslinks with DNA compared to PUVA, and therefore had a lower genotoxic potential (Vedaldi et. al., 1988; Morliere et. al., 1988; Riccio et. al., 1992). Furthermore, KUVA did not hyperpigment adjacent non-vitiliginous skin (Abdel-Fattah et. al., 1982; Honigsman et. al., 1985, Milne et. al., 1999), as was the finding with PUVA treatment. However, despite the above encouraging reports with KUVA treatment, dermatologists are not using this drug for vitiligo, since only a few *in vivo* and *in vitro* studies have been carried out with this drug.

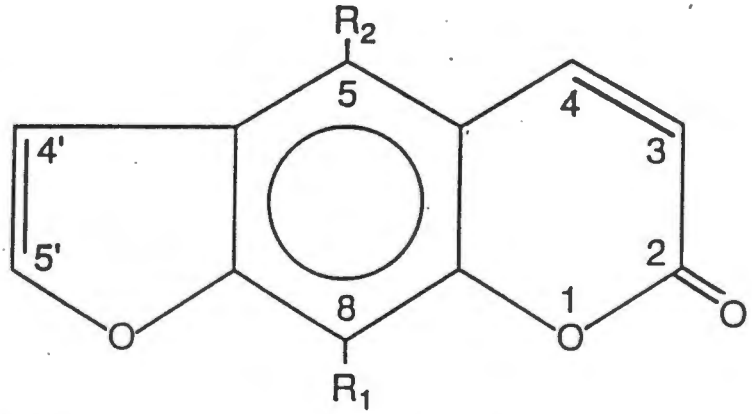
Khellin was used extensively in the 1940-1950's as a coronary vasodilator in the treatment of asthma and angina pectoris, until better treatments were discovered. No long-term adverse effects were reported in the ten years that this drug was used for asthma and angina, except for an increase in liver transaminases in certain patients. Thus, a liver complication was regarded as a contra-indication for the use of khellin. A possible explanation for this was, that khellin might have acted as an inhibitor of the microsomal cytochrome P-450 sub-enzyme of the liver, and was capable of inhibiting cytosolic enzymes (Schimmer and Rauch, 1998). The study by Bech-Thompsen and Wulf (1995), gave the first and only report of carcinogenicity with KUVA photochemotherapy *in vivo*. Tumours were formed after mice were treated with high UVA doses and 5% khellin cream, but no skin tumours were formed with low doses of UVA and 5% khellin cream. Since several *in vitro* results demonstrated that khellin formed cross-links with DNA in the presence of UVA, exposure to high UVA doses possibly resulted in tighter or more cross-links being formed, and therefore increased the potential for mutagenicity in these mice. In addition, UVA itself is well documented to be carcinogenic (Applegate and Frenk, 1995; Burren et. al., 1998), therefore the likelihood of high doses of UVA promoting carcinogenicity in these mice, is also probable. Despite the concentration of 8-MOP (in PUVA treatment) being four times lower than khellin (in KUVA

A



KHELLIN

B



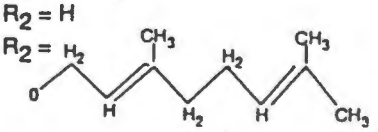
Psoralen	: $R_1 = H$	$R_2 = H$
5-Methoxypsoralen (5-MOP)	: $R_1 = H$	$R_2 = OCH_3$
8-Methoxypsoralen (8-MOP)	: $R_1 = OCH_3$	$R_2 = H$
5-Geranoxypsoralen (5-GOP) (bergamottin)	: $R_1 = H$	$R_2 =$ 

Figure 1.7: Molecular structures of KHELLIN (A, a furochromone) and PSORALEN (B, a furocoumarin). From Riccio et. al., 1992; Vedaldi et. al., 1988; modified from Kinley et. al., 1994.

treatment), more mice with tumours were produced with PUVA treatment in this study. This suggested that KUVA treatment was possibly safer and a better alternative treatment for vitiliginous skin than PUVA treatment.

The molecular mechanism(s) by which KUVA repigments vitiliginous skin are not known. Most of the studies done thus far, have been aimed at determining whether KUVA was a better agent than PUVA clinically. To establish whether KUVA was less mutagenic than PUVA, the potential of forming crosslinks with DNA *in vitro* by KUVA, was compared to that of PUVA in several studies. The genotoxicity of khellin was assessed in the following studies: Morliere et. al., (1988), found that khellin did not induce cross-links in the DNA of yeast cells. In contrast, it was found that khellin was able to form a few inter-strand crosslinks in the DNA of both Ehrlich ascites cells and a *Salmonella* strain (Vedaldi et. al., 1988; Riccio et. al., 1992). In this latter study, the inter-strand crosslinks formed within the *Salmonella* strain, occurred at psoralen concentrations 8-fold lower than the khellin concentration, and the UVA dose in PUVA treatment, was 13-fold lower than that used with KUVA treatment. Thus the notion that khellin may be safer than psoralens for clinical use, was highly supported. Trabalzini et. al. (1990), on the other hand, determined whether DNA damage, which may lead to mutagenicity, occurred by photo-oxidation of the DNA by KUVA, which was seen with PUVA treatment. It was revealed that KUVA had no photo-oxidising effect on the DNA. This study also provided further support for KUVA being less genotoxic than PUVA, and possibly a better therapeutic agent than PUVA.

There has been only one study that has provided some evidence for a possible mechanism of action of khellin and KUVA (di Stefano et. al., 1995). It was shown in the latter study that khellin alone and even more so KUVA, mediated its action via a receptor. In HeLa cells, it was shown that KUVA had an affinity for the Gi-protein-coupled pertussin-toxin sensitive protein receptors and also acted on adenylate cyclase. It is not known how repigmentation of the skin was brought about through these receptors, since no other study has investigated this finding any further.

While the above studies suggest that KUVA is a viable alternative treatment to PUVA, not much is known about the mechanism of action of KUVA in repigmenting vitiliginous skin. Indeed, it is not even known whether KUVA has a direct effect on the pigment cells itself or indeed whether it has a direct effect on the melanogenic pathway of these cells at all. Thus the *broad aim* of this study is to contribute towards the understanding of the mechanism(s) by which the khellin and UVA combination (KUVA), repigments the skin of vitiligo patients. The *specific aim* of this project was

to use an *in vitro* tissue culture system to evaluate the effects of the drug khellin, UVA and KUVA on melanocyte proliferation and pigmentation. The studies were carried out on cultured human melanoma cells, normal human melanocytes and 3T3 fibroblasts as the non-melanocytic control cell line. The following three specific questions were addressed in this project:

QUESTION 1: *What is the effect of khellin, UVA and KUVA on the proliferation of melanoma cells and normal human melanocytes?*

QUESTION 2: *Is the proliferative effect of khellin, UVA and KUVA pigment-cell specific?*

QUESTION 3: *What is the effect of khellin, UVA and KUVA on melanogenesis?*

CHAPTER 2

MATERIALS AND METHODS:

[2.1] CELL LINES / CELL CULTURE

2.1.1. UCT MEL-1

This cell line was obtained from Mr. G. Hanekom (Dept. of Clinical Science and Immunology, UCT). It was derived from a primary malignant tumour, which was excised from a patient at Groote Schuur Hospital, Cape Town (Hoal, E.G., Ph.D. thesis, 1981, UCT). Hoal (1981) described Mel-1 cells as displaying both dendricity and melanization throughout the passaging *in vitro*. The melanogenic state of later passages of Mel-1 cells was more recently characterized by Davids (Masters thesis, 1997, UCT). He demonstrated that these cells had an eumelanin content that was 3.3 times higher than that of the 3T3 fibroblasts, and showed that the rate of melanin formation of Mel-1 cells was 9 times higher than that of 3T3 fibroblasts. Furthermore, by means of Northern blotting and RT-PCR analyses, it was demonstrated that Mel-1 cells expressed TRP-1, TRP-2 and low levels of tyrosinase genes. The pigmented nature of Mel-1 cells is clearly demonstrated in Figure 2.1, where the colour of the cell pellets of Mel-1 cells, 3T3 fibroblasts and normal melanocytes is shown. This figure shows that Mel-1 cells are slightly less pigmented than the normal melanocytes, but clearly more pigmented than the 3T3 fibroblasts.

Melanoma cells were grown in RPMI-1640 medium, supplemented with 10% foetal calf serum (FCS) and 100IU/ml penicillin and 100mg/ml streptomycin. The medium was changed twice weekly. For all the proliferation studies, the cells were lifted with trypsin/EDTA (0.02% trypsin/0.05% ethylenediamine tetraacetic acid) and $1x10^4$ cells were plated in triplicate into wells of microtiter dishes. Cells were counted as described in Section 2.3.1.1

2.1.2. 3T3 FIBROBLASTS

3T3 fibroblasts were grown under standard culture conditions. The cells were cultured in RPMI-1640 medium supplemented with 10% FCS and 100 IU/ml penicillin and 100 mg/ml streptomycin, in a 37°C humidified 5% CO_2 atmosphere. Sub-confluent cell cultures were always maintained to

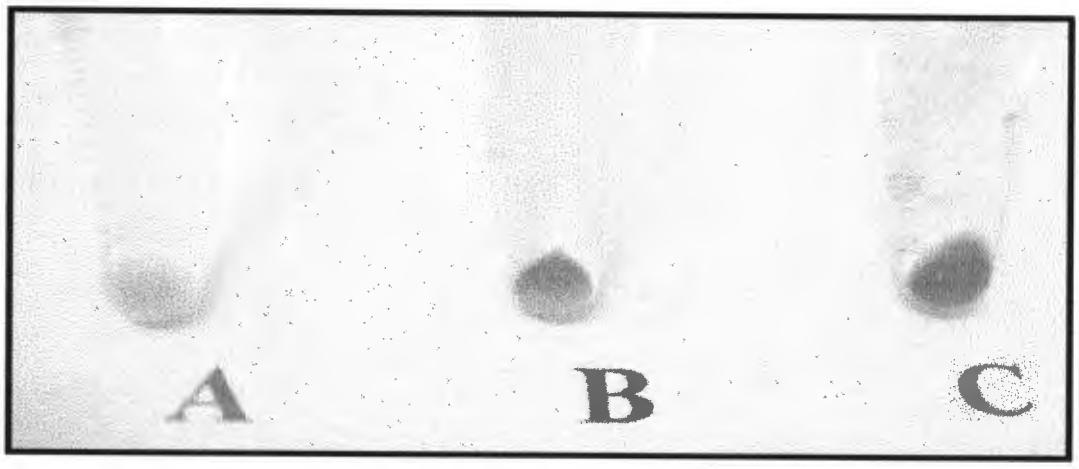
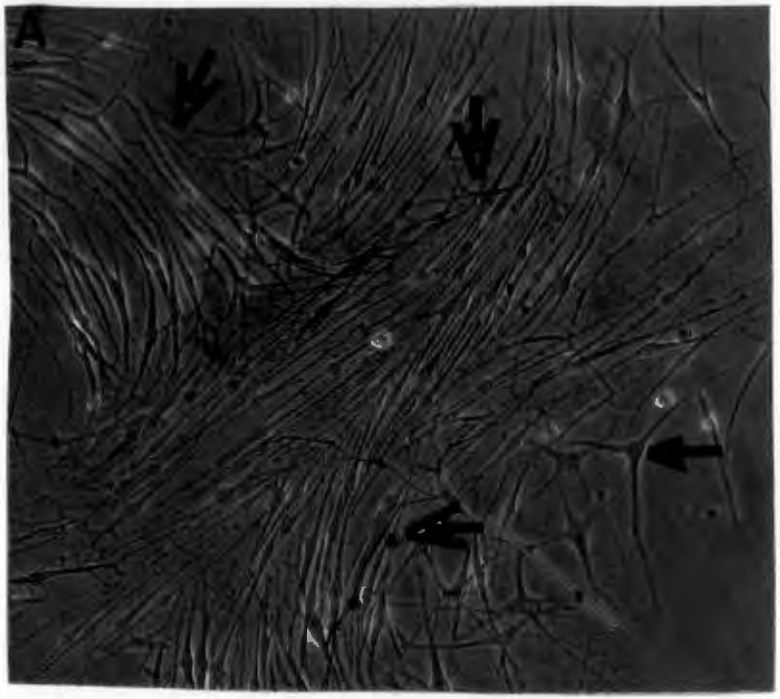


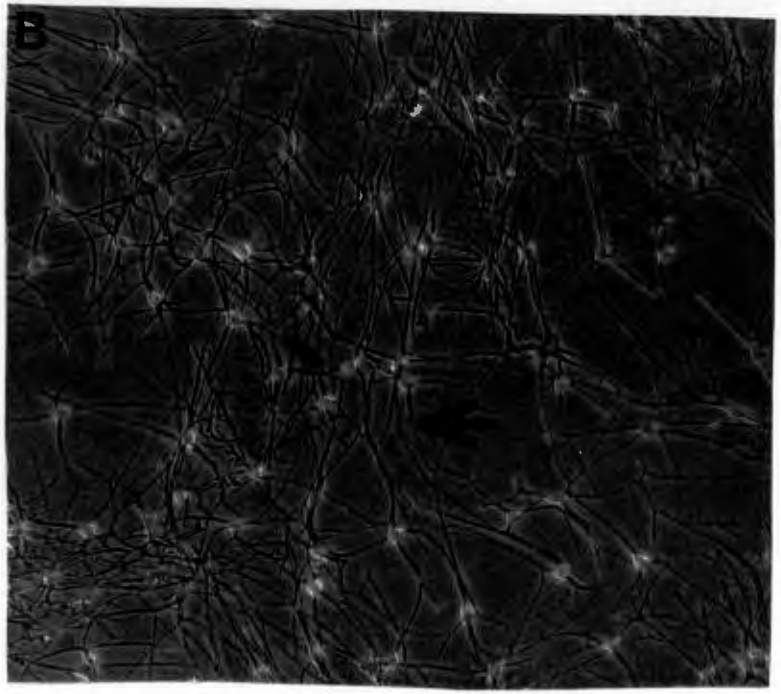
Figure 2.1: Increased pigment density of cell pellets. After trypsinization, each cell suspension was pelleted in a tube by centrifugation of 2.500g for 5 minutes. The cells are represented in the photograph above as: A, 3T3 fibroblasts; B, Mel-1 melanoma cells; C, normal melanocytes

prevent the formation of variants. For the proliferation studies, $5x10^3$ cells were plated in triplicate into wells of microtiter dishes.

2.1.3. NORMAL HUMAN MELANOCYTES

Normal human melanocytes were obtained by a modified procedure previously described by (Halaban et. al., 1984; Eisinger et. al., 1982). In short, normal human melanocytes were cultured from neonatal and adult foreskins of routine circumcision, courtesy of Dr. Ebrahiem, a general surgeon at Gatesville Medical Centre (South Africa), and foreskins were also obtained from Dr. N.A Parker, a general practitioner. The initial cultures of normal human melanocytes were grown from human skin samples, which were obtained from Dr. Dommisse, a plastic surgeon at Claremont Hospital (South Africa). These cultures were not used for experimental work, due to the low yield of cells and the fact that these cells proliferated much slower than neonatal melanocytes. All the processing was carried out with sterile instruments and under sterile culture conditions. The skin samples were transported on ice in Ham's F10 medium, supplemented with 1% penicillin/streptomycin and 0.1% Fungizone. The skin samples were washed in phosphate-buffered saline (PBS, pH 7.4, see appendix), were cleansed of excess subcutaneous tissue, cut into small pieces (5 x 5 mm) and incubated overnight at 4°C in trypsin/EDTA solution [0.25% trypsin / 0.05% EDTA in PBS (pH 7.4), 1% penicillin/streptomycin and 0.1% Fungizone]. The epidermis was teased apart from the dermis with a fine forceps, and then the dermis was discarded. The epidermal sheets were dissociated by manual fragmentation and then triturated in a 1ml Gilson tip or vortexed into a single cell suspension as described by Eisinger et. al. (1982). The cell suspension was added to a petri dish and then cultured in Buffalo rat liver (BRL)-conditioned medium, (see Appendix and paragraph 2.1.4 for details), which was supplemented with 20% FCS, 16mM TPA, 0.1mM IBMX, 100IU/ml insulin, and 1% cholera toxin. This medium preferentially supported the growth of human melanocytes. The cells were left at 37°C in a 5% CO_2 humidified chamber overnight and allowed to adhere. The following day, the medium containing dead cells and other cell debris was sucked off and fresh medium was added to the dishes. When contamination of fibroblasts or keratinocytes was suspected after visual inspection of the dishes with a phase contrast microscope, Geneticin (100μg/ml, Sigma) was added daily to the culture dish for up to seven days if necessary (Halaban et. al., 1984). The cells were continually cultured at 37°C in a 5% CO_2 atmosphere, and the medium was changed every alternate day for the first two weeks and twice weekly thereafter. Second passage cells at 70% confluency, were used as material for evaluating the effects of khellin,





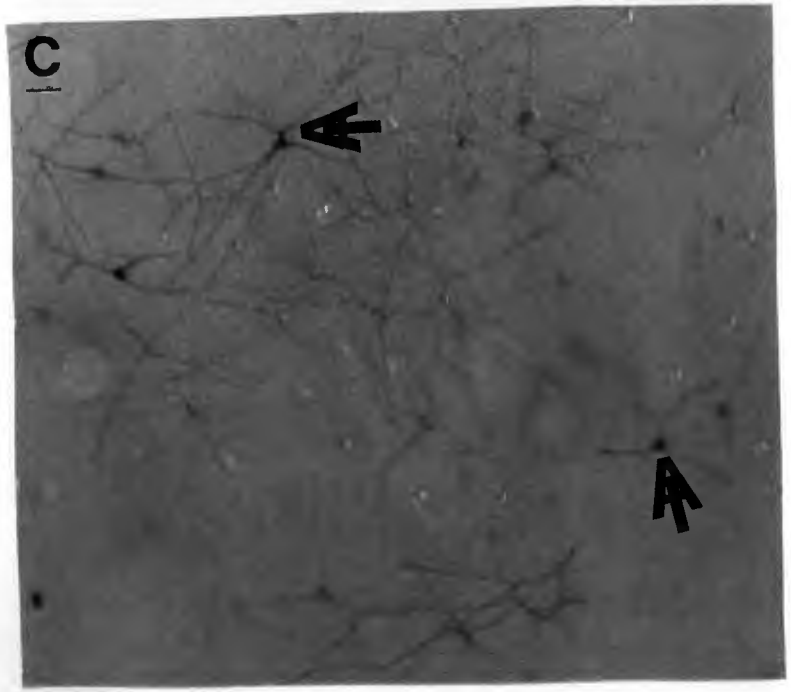


FIGURE 2.2 Photomicrograph of semi-confluent cultures of Normal Human Melanocytes, derived from neonatal foreskins.
(A) Phase Contrast: A typical culture undergoing proliferation as seen by the parallel array of the cells (see arrows). Melanocytes appear as single bipolar or polydendritic cells (see arrows). Pigment granules are even visible in the cell bodies of a few cells under phase contrast.
(B) Phase contrast photomicrograph showing dendritic morphology. Dendrites extending in all directions (see arrows)
(C) Bright field: Photomicrograph shows pigment granules more clearly in cell bodies and dendrites of melanocytes (see arrows)

UVA and KUVA on normal human melanocytes. For the proliferation assays, the cells were lifted with trypsin/EDTA (0.02% / 0.05%) and plated in triplicate at a density of $2x10^4$ cells/ml into wells of the microtiter dishes. See Figure 2.2 (a, b and c).

2.1.4. BUFFALO RAT LIVER CELLS

Buffalo-rat liver cells are a source of steel factor (Zsebo et. al., 1990), which is essential for the maintenance, survival, proliferation and differentiation of normal human melanocytes (Kunisada et. al., 1998). These cells were grown to confluence in Ham's F10, 20% FCS and supplemented with 100IU/ml penicillin / 100 mg/ml streptomycin and 1mM L-glutamine at 37°C in 5% humidified CO_2 atmosphere. The medium was changed twice a week until the dish was confluent. From then onwards, the medium was collected every day. This collected medium contained the steel factor produced by the BRL cells. BRL-conditioned medium was then filter-sterilized with a 0.45μm filter before supplements were added (see Appendix).

2.1.5. HOECHST STAINING

Before any of the experiments were commenced, cultures were tested for the presence of mycoplasma contamination. Mycoplasma is a type of bacteria, which is not visible microscopically. It has no cell wall, and since the antibiotics, penicillin and streptomycin, act by inhibiting cell wall synthesis (Goodman et. al., 1990), the antibiotics are thus ineffective for this type of infection. Because mycoplasma contain DNA, the fluorescent bisbenzimidazole dye, Hoechst 33342 (see Appendix), can bind to this DNA after it penetrates the plasma membrane. Therefore, mycoplasma-contaminated cultures could be detected under a fluorescent microscope by their characteristic particulate or filamentous pattern of fluorescence on the cell surface, or the surrounding cell cytoplasm if the contamination was heavy. Furthermore, since mycoplasm take up thymidine from the culture medium, infected cultures would show signs of deterioration or a decrease in cell proliferation could occur. No mycoplasma infections were observed in any of these studies.

2.2.1. CHEMICALS

Before the experiments described below were commenced, it was necessary to first find the most ideal vehicle for khellin. This very problem of finding the most appropriate vehicle for dissolving pigment cell modulators, was recently addressed in the paper of Virador et. al. (1999). It was found that khellin (Sigma) displayed poor solubility in water, PBS, absolute ethanol and 70% ethanol. Thus a variety of solutions were tested as vehicles for khellin (see Chapter 3, page 40, paragraph 1 of Results). It was concluded that DMSO (di-methylsulfonamide, BDH) was the most ideal vehicle for the dissolution of khellin. For the experiments described below, khellin was dissolved in the vehicle, DMSO, and was stored as a 100mM sterile stock solution at 4^0C, protected from light with foil. In addition, khellin solutions were added to the cells in a dark sterile hood. The necessary dilutions were made with the culture medium to obtain the appropriate khellin concentration. A concentration of less than 1% DMSO, was maintained throughout the experiments. Growth curve studies of Mel-1 melanoma cells treated with various doses of DMSO in culture medium revealed that DMSO slightly inhibited the growth rate of melanoma cells. However, the results were expressed relative to the control, which was also treated with DMSO.

2.2.2. CULTURE PLATING PROCEDURES

After lifting the cells from the dish, they were inoculated at a density of $1x10^4$ and $5x10^3$ cells per well of both Mel-1 and 3T3 fibroblasts, respectively. Similarly, normal human melanocytes were plated at a density of $2x10^4$ cells per well. The cells were allowed to attach overnight to the culture dish in the standard culture medium. The next day, the melanocytes were exposed to the respective experimental treatment procedures. 3T3 fibroblasts served as a negative cell line for all experiments. The cells were plated in triplicate and all experiments were repeated at least thrice.

2.2.3. EXPOSURE OF CELLS TO KHELLIN ALONE

The medium of the cells was replaced with various concentrations of khellin. The cells were exposed to khellin for four days, and thereafter, assessment of results was made. All experiments were repeated at least thrice, and using different batches of cells if at all possible. The cells were exposed initially to a wide concentration range of khellin, and a more optimal dose range was then discovered. This dose range was used for the experiments that followed.

were repeated at least thrice, and using different batches of cells if at all possible. The cells were exposed initially to a wide concentration range of khellin, and a more optimal dose range was then discovered. This dose range was used for the experiments that followed.

2.2.4. EXPOSURE OF CELLS TO UVA ALONE

Two days after plating, melanoma cells were exposed to UVA light with doses ranging from 0 – 480mJ/cm^2. An optimal proliferative UVA dose was then discovered and used for all subsequent experiments. Exposing cells to UVA light involved the following:

(i) Exposure to UVA light took place in a customized UVA cabinet, fitted with UVA lamps (wavelength of 365nm). The energy was measured with a Waldmann Lichttechnik radiometer, intensity of 0.8mW/ cm^2. The doses of UVA were determined by using the following formula: Dose (mJ/cm^2) = radiometer reading (mW/cm^2) x time in seconds. A standard curve was used to determine the time of UVA exposure for a particular dose to be delivered to the cells experimentally.

(ii) The cells were plated into the dish, which was placed at a standard 30cm distance from the UVA source for all the UVA experiments.

(iii) To exclude any UVB rays, a 4mm plate glass was placed over the lid of the dish, which served as a UVB filter. In addition, the customized UVA cabinet was painted black to prevent reflection of UVA rays, which could affect the actual UVA dose delivered to the cells.

(iv) The dish with cells was placed in an ice bucket to prevent detachment of cells due to the heat energy released during the irradiation procedures.

(v) Medium was aspirated from the dishes before the irradiation procedure, in order to avoid the formation of medium-derived toxic photo-products, which could be induced after UV exposure (Kao and Yu, 1992), and were irradiated in the presence of PBS.

(vi) 48 hours after seeding of the cells, the cells were exposed to 30mJ/cm^2 - 480mJ/cm^2 of UVA light.

(vii) Immediately after irradiation, PBS was aspirated and replaced with the standard culture medium. The dish was then placed at 37^{0}C, in a humidified 5% CO_2 atmosphere for four days. The medium was changed every two days.

(viii) The control cells were covered with a black cloth for the entire irradiation procedure within the UVA cabinet (called sham-irradiation).

2.2.5. EXPOSURE OF CELLS TO KUVA (KHELLIN COMBINED WITH UVA) TREATMENT

The same precautions as mentioned in sections 2.2.3 and 2.2.4 were followed during the KUVA treatment protocol.

One day after seeding the cells and one day before the phototreatment, normal medium was replaced with medium containing 0.01mM, 0.1mm and 1mM khellin, and the cells were incubated overnight. The next day, medium was replaced with pre-warmed PBS and exposed to a single dose of 250mJ/cm^2 UVA light. The sham-irradiated cells, treated with or without khellin, were covered with a black cloth and were also present in the UVA irradiation cabinet during the irradiation procedure. Immediately after irradiation, the PBS was replaced with basal medium containing 0.01mM, 0.1mM and 1mM khellin into the appropriate wells. Responses were measured after a further four days of cell culturing in the presence of khellin, and medium was changed every two days.

2.2.6. ASSESSMENT OF RESULTS

Four days after the experimental exposure of cells, the results were assessed by the method described below under the sections: proliferative assay, melanin formation assay and western blotting.

[2.3] ASSAY PROCEDURES

2.3.1. Cell Proliferation Assay

Initial attempts were made to measure proliferation with ^{3}H-thymidine incorporation (see Appendix) and a XTT cell proliferation kit (Boeringer Mannheim). However, the small quantities and slow growth rate of the normal human melanocyte cultures, rendered these methods unsuitable. Therefore, cell proliferation was assessed by counting.

2.3.1.1. Cell Counting Protocol

After the cells in the microtiter dishes were treated with khellin, UVA or KUVA, the medium was aspirated, and the cells were washed twice with 300µl PBS (pH 7.4). The cells were removed from

the dish, after the addition of 50µl of trypsin/EDTA at room temperature to each well (2 – 4 minutes). 150 µl of medium supplemented with FCS was added to inactivate the enzyme. The cells were triturated with a Gilson tip set at 200µl, before being placed into Eppendorf tubes, which were centrifuged at 2,500 g for 10 minutes at room temperature in a bench top centrifuge. 150µl of the supernatant was removed and the pellets were re-suspended in the rest of the supernatant (50µl). The cell suspension was then vortexed for 5 seconds, followed by cell counting, using a haemacytometer. Each experiment was performed in triplicate, and the entire experiment was repeated at least thrice. The initial experiments were done in conjunction with the trypan-blue exclusion dye assay. The result recorded is a reflection of only viable cell numbers counted.

[2.3.2] Cytotoxic Assay

Cells were incubated in the presence of khellin (0-1mM) alone, as described above (2.2.3) for four days. Trypan-blue exclusion dye assay was used to count both the viable and non-viable (dead) cells. The results are recorded as the number of viable cells, which is expressed as a percentage of the total number of cells (number of both viable and dead cells) in the culture dish of the experiment. The lower the percentage of viable cells for example, the higher the percentage of dead cells. This experiment was only done once and was carried out in duplicate.

[2.4] PROTEIN DETERMINATION

Protein assays were carried out using either the BCA (bicinchoninic acid assay, Pierce) assay kit or the Bio-rad protein assay. (See appendix)

[2.5] *IN VITRO* RADIOMETRIC ASSAYS

2.5.1. MELANIN FORMATION ASSAY:

The melanin formation assay measures the incorporation of ^{14}C-tyrosine into acid insoluble melanin, and gives a measure of the melanocyte-specific enzyme activities of the entire melanin-forming pathway (Hearing and Ekel, 1976). The cells were exposed to khellin alone, UVA alone and KUVA as described earlier, before melanin formation assays were carried out. The protein concentrations of the cell lysates used in these assays were standardised to the lowest protein

concentration, before each assay (see Appendix) was performed. Each assay solution (final volume 25µl) contained:

10µl enzyme preparation or 10µl 0.1M phosphate buffer only (control sample), 5µl L-(U-^{14}C)-tyrosine {25(Ci/ml, specific activity 513mCi/mmol., The Radiochemical Centre, Amersham}, a 5µl of a solution containing 1mg/ml chloramphenicol, 1mg/ml cycloheximide, 1000IU/ml penicillin-G and 0.1mg/ml bovine serum albumin, and 5µl 3,4-dihydroxy-L-phenylalanine (0.05mM in 0.1M PBS (pH 7.4).

The assay samples were incubated in triplicate for three-and a half-hours at 37^{0}C. 20µl of each sample was pipetted onto GF/C glass microfibre filter discs. The discs were then treated as follows: three washes with 150ml ice-cold 10% TCA (Trichloroacetic acid, see Appendix), one 5 minute wash with 96% (^{v}/v) ethanol, and one 5 minute wash in acetone which removed unwanted cellular debris from the discs. This was followed by transfer of the filters to scintillation vials, and air-drying for 1 hour at room temperature or 30 minutes at 37^{0}C. The precipitated proteins on the filters were then solubilised by the addition of 200µl Soluene-350 (Packard) onto the filters, and the vials were incubated at 37^{0}C for 1 hour. This was followed by the addition of 7µl of glacial acetic acid to neutralise the Soluene-350, to reduce chemiluminescence during scintillation counting (Zinsser Analytic, Germany, manufacturer's instructions, personal communication). Finally, 10ml Quicksafe scintillation fluid (Packard) was added to each vial, and ten minute counts were performed in a Beckmann liquid scintillation counter. The controls used were blanks from which the enzyme was omitted. Each assay was expressed as percentage cpm/µg of protein and as a percentage of the experimental untreated control.

[2.6] WESTERN BLOTTING

After the cultured cells were exposed to KUVA, the cells were harvested and solubilized overnight at 4^{0}C in CEB (see Appendix), then centrifuged at 12000g for 15 min at 4^{0}C, and the supernatants were then recovered. Protein concentrations were determined with the BCA assay (Pierce) and Bio-rad assay (Bio-rad laboratories) kits, and bovine serum albumin was used as the standard. Equal amounts of protein (40µg) were loaded in each lane of a 7.5% SDS poly-acrylamide gel, and electrophoresed under reducing conditions, after which proteins were transferred onto Hybond ECL nitrocellulose membranes (Amersham). Following blocking for 4 hours at room temperature (RT) in 10% non-fat dry milk powder in TBS/Tween (0.1% Tween-20 in Tris buffered saline), the blots

were incubated with the following rabbit antisera, which were obtained as gifts from Dr Vincent Hearing, National Cancer Institute): α- PEP8, a polyclonal antibody against the carboxy terminus of mouse TRP-2, 1:500 dilution in 5% milk powder in TBS-T; α-PEP7, a polyconal antibody against the carboxy terminus of tyrosinase; 1:500 dilution in 5% milk powder in TBS-T, overnight at room temperature. The membranes were then washed twice with TBS-T for 20 minutes and incubated with horseradish peroxidase-conjugated swine-anti-rabbit IgG secondary antibody (1:1500 dilution in TBS-T) for 30 minutes at RT. After washing the membranes four times with TBS-T, the immunoreactive proteins were detected by Enhanced Chemiluminescence (Amersham), according to the manufacturer's instructions.

[2.7] STATISTICAL ANALYSIS

The analyzed results were a reflection of at least triplicate samples, unless otherwise indicated, from various groups of experiments. The data of the results were represented as the mean +/- S.E.M. in the whole study. To determine the significance of the results, a Student t-test was performed between group comparisons. A 95% confidence interval was set and therefore, $p < 0.05$, was considered statistically significant.

[2.8] CALCULATION OF POPULATION DOUBLING (From Celis, 1991)

The population doubling of a culture was calculated directly from the cell count numbers. The increase in population doublings was calculated by the following formula: $N_H / N_1 = 2^X$, where N_H = the number of cells harvested after treatment and N_1 = the number of cells inoculated, and X= the number of population doublings. Another way to present this calculation is as follows:

$$\frac{\text{Log}\,(N_H) - \text{Log}\,(N_1)}{\text{Log}\,2} = X$$

CHAPTER 3

RESULTS:

Dissolution of khellin:

In order to carry out studies on the effect of khellin on melanocyte proliferation and melanogenesis, it was first essential to establish the best way to dissolve the drug in the tissue culture medium. Previous investigators have used a variety of vehicles for dissolving khellin, including, absolute ethanol (Kinley et. al., 1994; di Stefano et. al., 1988), Tris buffer pH 7.4 (Morliere et. al., 1988) and other aqueous solutions (Vedaldi et. al., 1988), for *in vitro* studies. In addition, oil-in-water emulsions (aqueous cream, Honigsman et. al., 1985, Milne et. al., 1999), acetone and propylene glycol (Orrechia et. al., 1992), were used *in vivo*. In the present study water, PBS, absolute ethanol, 70% ethanol and DMSO were tested as possible vehicles. It was found that DMSO was the only vehicle that dissolved the drug effectively. To determine whether DMSO would affect the proliferation of Mel-1 cells and 3T3 fibroblasts, the cells were exposed to a range of DMSO concentrations. It was found that DMSO was cytotoxic to both Mel-1 cells and 3T3 fibroblasts, especially at concentrations above 1% ^{v}/v. Thus, for all the experiments that followed, DMSO was used at a maximum concentration of 1% ^{v}/v.

All experiments were repeated at least three times, except where otherwise indicated.

Khellin stimulates the proliferation of human melanoma cells and normal human melanocytes (NHMs), but inhibits the proliferation of fibroblasts.

To determine whether khellin stimulates cell proliferation, assays were carried out on Mel-1 melanoma cells and 3T3 fibroblasts exposed to khellin for 4 days over a concentration range from 1nM (10^{-9} M) to 1mM (10^{-3} M). As seen in Figure 3.1, khellin stimulated proliferation of Mel-1 cells over a broad range of khellin concentrations, from 1nm to 0.1mM, with maximal stimulation at 0.01mM (10^{-5} M). At this maximal dose, after four days, the total number of cells in the dishes was increased 2.35-fold above the untreated control. In contrast to the results of the Mel-1 melanoma

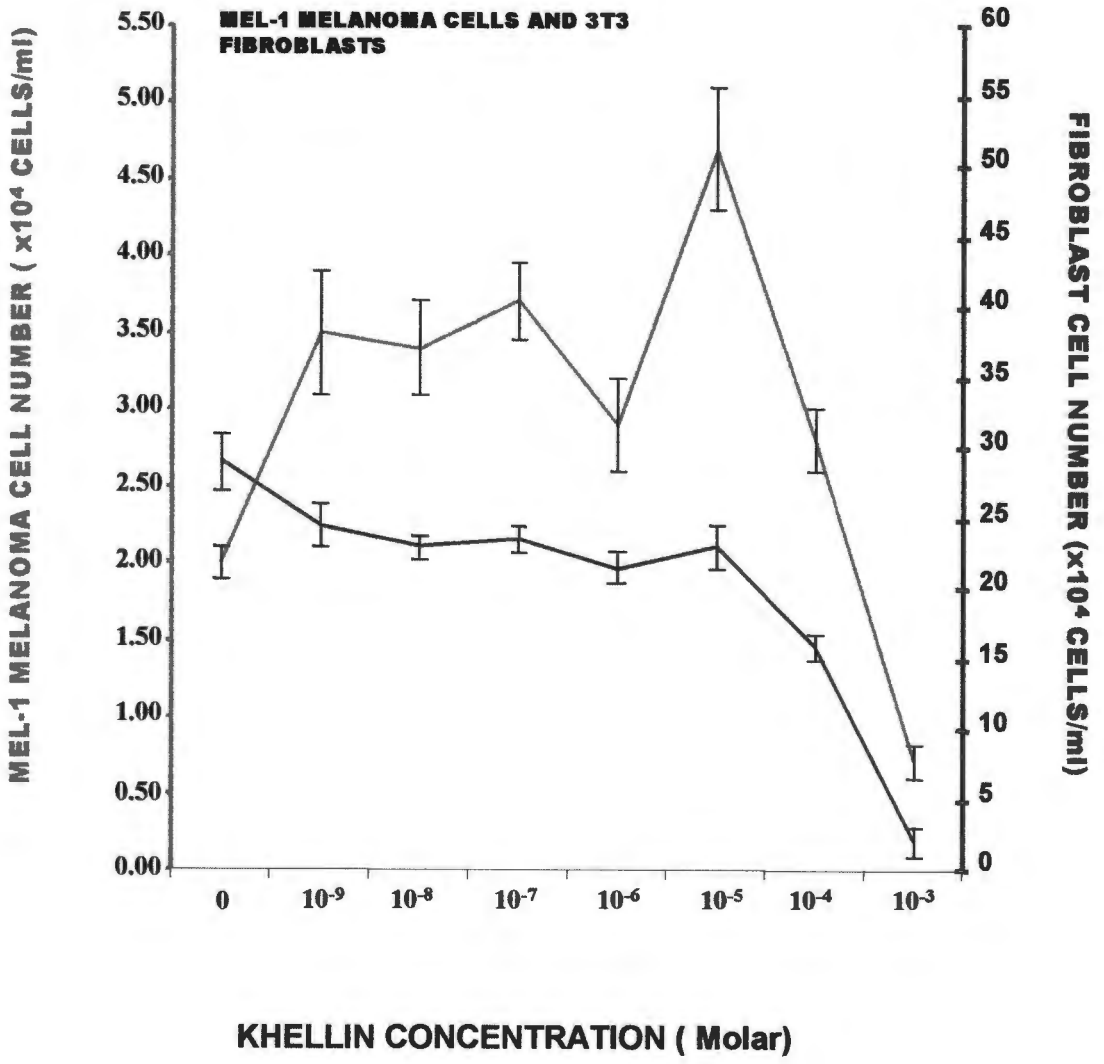


FIGURE 3.1: Effect of Khellin (1nM-1mM) on Proliferation of Mel-1 Melanoma cells and 3T3 Fibroblasts. Growth profiles of Mel-1 melanoma cells (orange line) and 3T3 mouse fibroblasts (purple line) cultured in khellin medium for four days. The data depicted in this figure are presented as the mean number of cells ± S.E.M. of at least five separate experiments, each performed in triplicate.

cells, exposure of 3T3 fibroblasts to khellin resulted in a decrease of the total number of cells in the dishes, over the entire concentration range tested (1nM–1mM) (Figure 3.1).

To obtain a better understanding of the kinetics of khellin enhanced proliferation of Mel-1 melanoma cells, time course assays over a four day period were carried out (Figure 3.2). The results revealed that even at day 1, khellin was stimulatory at doses below 0.5mM. At day 4, an even more pronounced stimulatory proliferative effect was obtained at doses below 0.5mM. Maximum proliferation was observed with 0.01mM khellin, which confirmed previous observations. At this dose, a 2.1-fold increase in the growth rate of Mel-1 cells was obtained, after four days of treatment.

In all the above experiments, the numbers of cells per dish were reduced when they were treated with khellin at concentrations of 0.5mM and above. This suggested that these higher doses of khellin had a cytotoxic effect on the melanoma cells. To test this hypothesis, cytotoxic assays with trypan-blue dye exclusion were carried out on melanoma cells treated with 0.01mM, 0.1mM and 1mM khellin (see Figures 3.3 [a] and [b]). The results from these assays suggest that 1mM khellin is indeed cytotoxic and not merely cytostatic to the melanoma cells (see Figure 3.3a). Thus, for example, at day four, only 28% of Mel-1 cells were able to exclude trypan blue (remained viable) compared to the control dish, wherein 100% of the cells were viable. Addition of 0.01mM and 0.1mM khellin, in contrast, resulted in more than 98% of the melanoma cells remaining viable. These results clearly demonstrate that the higher doses of khellin are cytotoxic and that lower doses are not cytotoxic and stimulate proliferation of melanoma cells.

The effect of khellin on the proliferation of 3T3 fibroblasts was also determined in this study (Figure 3.4). It was found that khellin reduced the total number of 3T3 fibroblasts per dish. This reduction was seen over the entire concentration range tested. To determine whether this decrease in cell numbers was due to the cytotoxicity of the drug, cytotoxic assays were also carried out on 3T3 fibroblasts that were treated for four days with khellin (0-1mM). The results revealed that only 22% of the fibroblasts were able to exclude trypan blue after four days of treatment with 1mM khellin (Figure 3.3b). These results suggest that at 1mM, khellin is cytotoxic to the fibroblasts. Furthermore, the results indicate that 1mM khellin is as cytotoxic to the fibroblasts as it is to the melanoma cells. Analysis of the effect of lower doses of khellin on fibroblasts revealed a slightly different picture compared to the melanoma cells. These results suggested that 0.1mM khellin was more cytotoxic to the fibroblasts than the melanoma cells. Interestingly, 0.01mM khellin did not

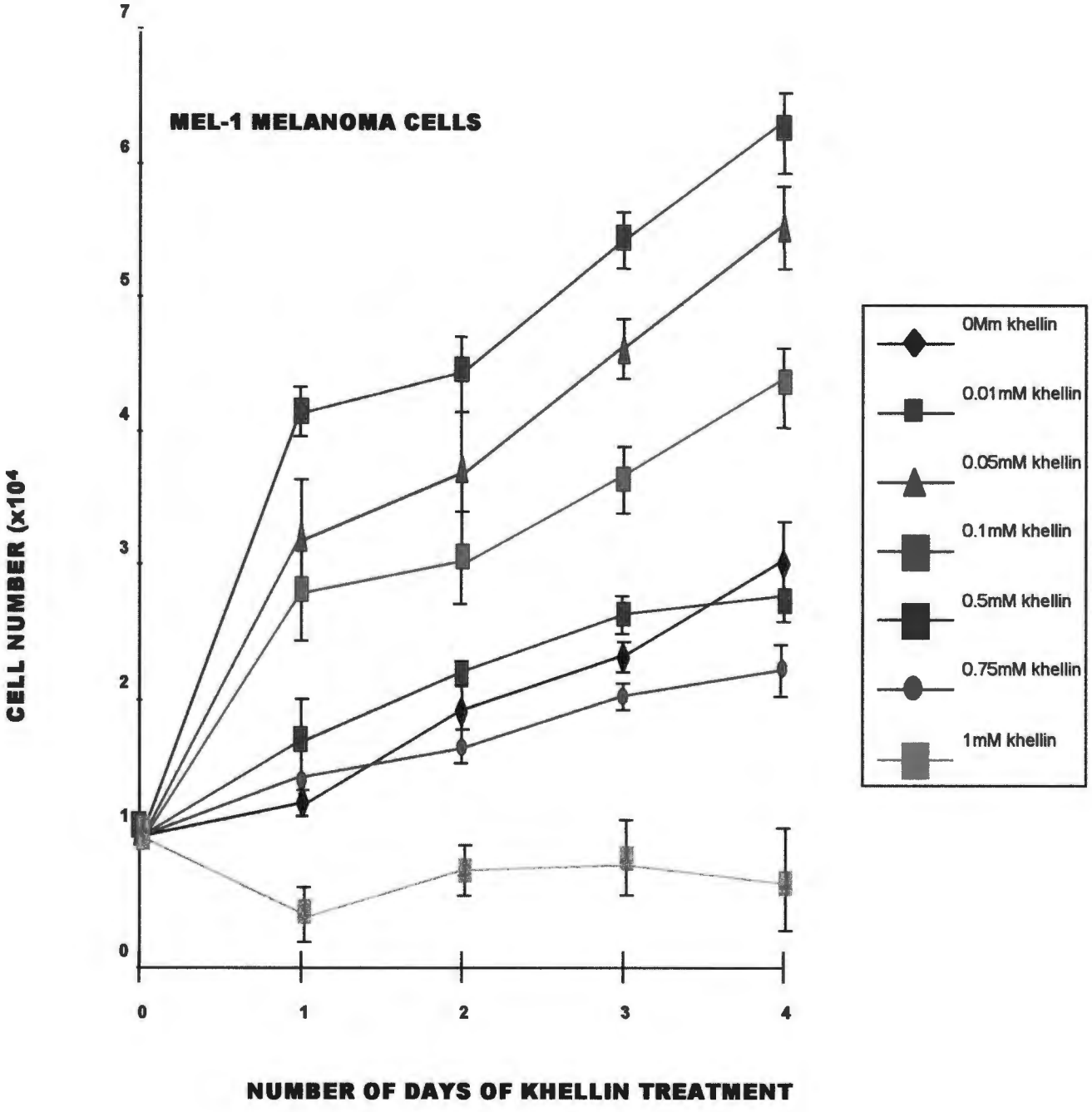


Figure 3.2: Effect of Khellin (0.01mM - 1mM) on Proliferation of Mel-1 Melanoma cells. Growth profile of Mel-1 melanoma cells grown in the presence of khellin, with different colours representing the various concentrations, over a time course period of four days. Control cells were grown in RPMI 1640 medium with 1% DMSO and with no khellin. Data are presented as the mean cell number ± S.E.M. of at least 6 separate experiments, and each performed in triplicate.

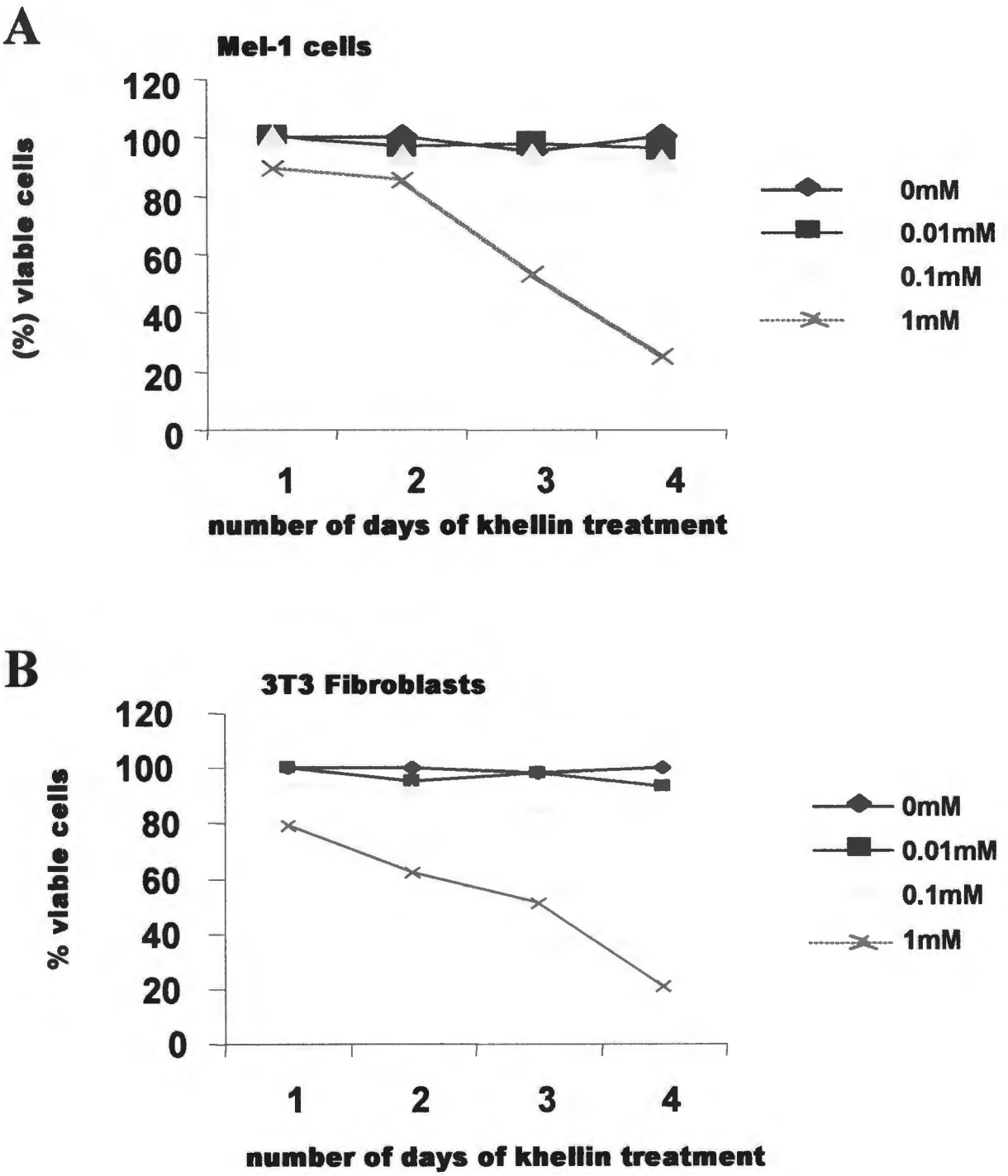


Figure 3.3 a, b: Percentage Viable Mel-1 cells (A) and 3T3 Fibroblasts (B) treated with Khellin (0 - 1mM). Cells were cultured in the absence of khellin (blue diamonds) or in the presence of 0.01mM (purple squares), 0.1mM (yellow triangles) or 1mM (light blue asterisks) khellin for four days. The data are representative of one experiment, which was performed in duplicate.

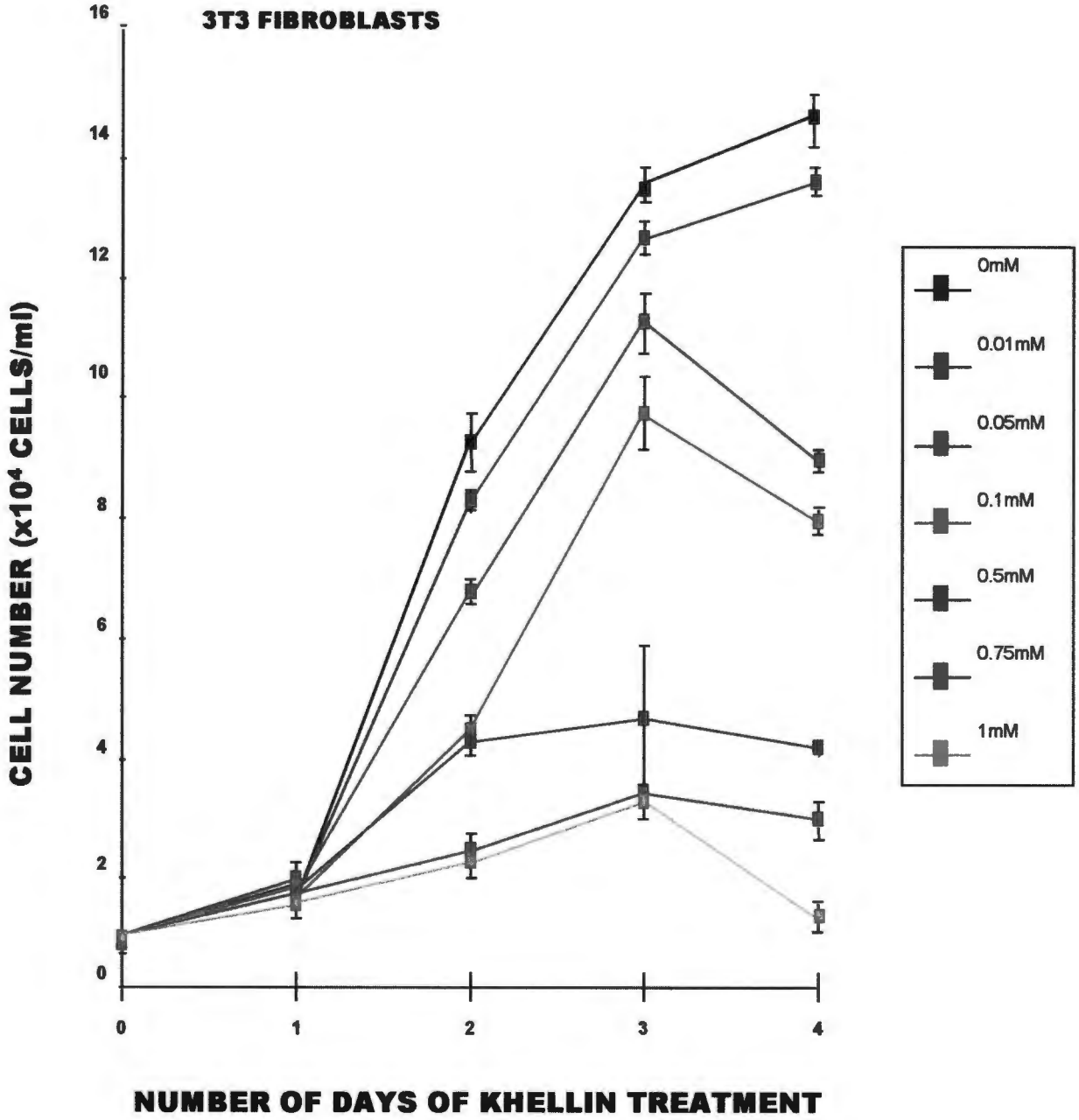


Figure 3.4: Effect of Khellin (0.01mM - 1mM) on Proliferation of 3T3 Fibroblasts. Growth profile of 3T3 mouse fibroblasts grown in the presence of khellin, with different colours representing the various concentrations, over a tine course period of four days. Control cells were grown in RPMI 1640 medium with 1% DMSO and with no khellin. Data are presented as the mean cell number ± S.E.M. of at least 6 separate experiments, each performed in triplicate.

appear to be significantly cytotoxic to the fibroblasts, even though a decrease in the cell numbers of fibroblasts was observed in the cell proliferation assays (Figure 3.4). These results suggest that at the lower doses of khellin, the drug is cytostatic, whereas the higher doses of khellin, is cytotoxic to the fibroblasts. Doses higher than 1mM khellin were not tested in this study.

Establishment of normal human melanocyte cultures

In order to investigate the effect of khellin on normal human melanocytes (NHMs), it was necessary to establish cultures of melanocytes from skin biopsies. Cultured melanocytes were derived from foreskins of circumcised infants of different races. Most times, melanocytes from different racial groups were cultured together and then used for experiments. Though this was clearly not ideal, it was necessary to do this in order to obtain large numbers of melanocytes that were needed for the experiments. In the present study, NHMs were found to have a decrease in their proliferation rate after about 4-5 weeks in culture. Thus, cultures of about two weeks old were always used to ensure that cells were proliferative when treated.

Because of the difficulties in obtaining large quantities of NHMs and the scarcity of material, studies on cultured normal human melanocytes were carried out only at concentrations of khellin that were shown to have maximal stimulatory effect (0.01mM and 0.1mM khellin) and cytotoxic effect (1mM) on Mel-1 melanoma cells. In dishes treated with 0.01mM and 0.1mM khellin, the total number of cells increased by 1.8-fold and 1.4-fold, respectively (Figure 3.5). T-tests revealed that the differences were significant at the $p < 0.05$ level, compared to the untreated control cells. Although it was not shown in this figure, khellin also inhibited the proliferation of the fibroblasts, as previously reported (Figures 3.1, 3.4).

UVA stimulates the proliferation of human Mel-1 melanoma cells, but had no effect on the proliferation of normal human melanocytes and fibroblasts.

To determine the effect of UVA on proliferation, Mel-1 cells and 3T3 fibroblasts were exposed to a single dose of UVA irradiation, in the dose range of 30 - 480mJ/cm^2, as described in Materials and Methods (Figure 3.6). After exposure to UVA doses of less than 150mJ/cm^2, no significant difference in the proliferation of the Mel-1 cells, compared to the sham-irradiated (control) cells, was seen. However, proliferation was enhanced at doses between 150-280mJ/cm^2 UVA, with a maximal stimulation at 250mJ/cm^2. At this maximal dose, proliferation was increased by 2.1-fold above the sham-irradiated control cells. For all subsequent experiments, a UVA dose of 250mJ/cm^2

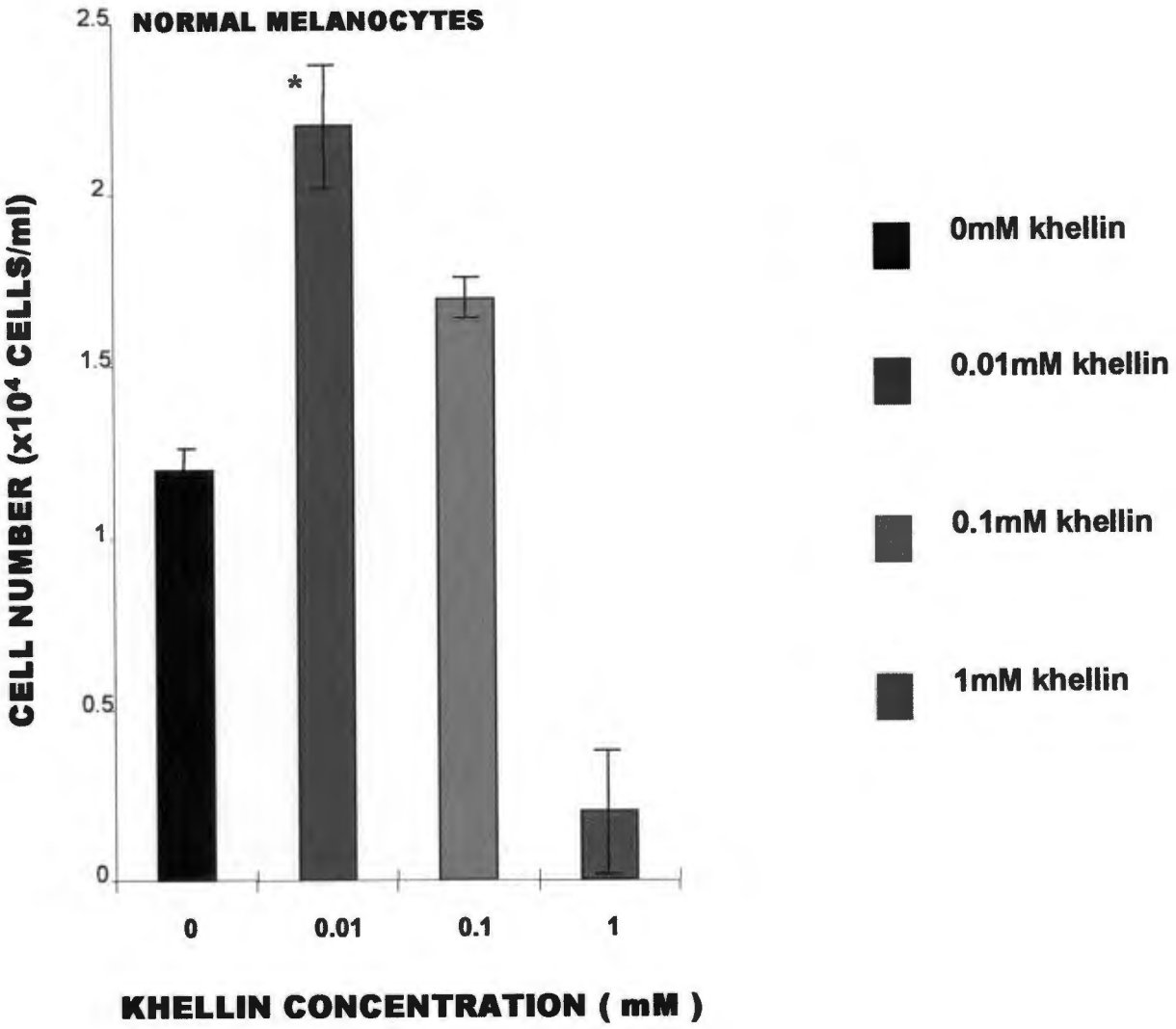


Figure 3.5: Effect of Khellin (0.01mM - 1mM) on Proliferation of Normal Human Melanocytes. Growth profiles of normal human melanocytes treated with khellin over a four-day time course period. The control cells were cultured only with medium and 1% DMSO. The data represented is the mean number of cells ± S.E.M of at least 4 separate experiments, each performed in triplicate. * $P < 0.05$ (result significant), versus control cells.

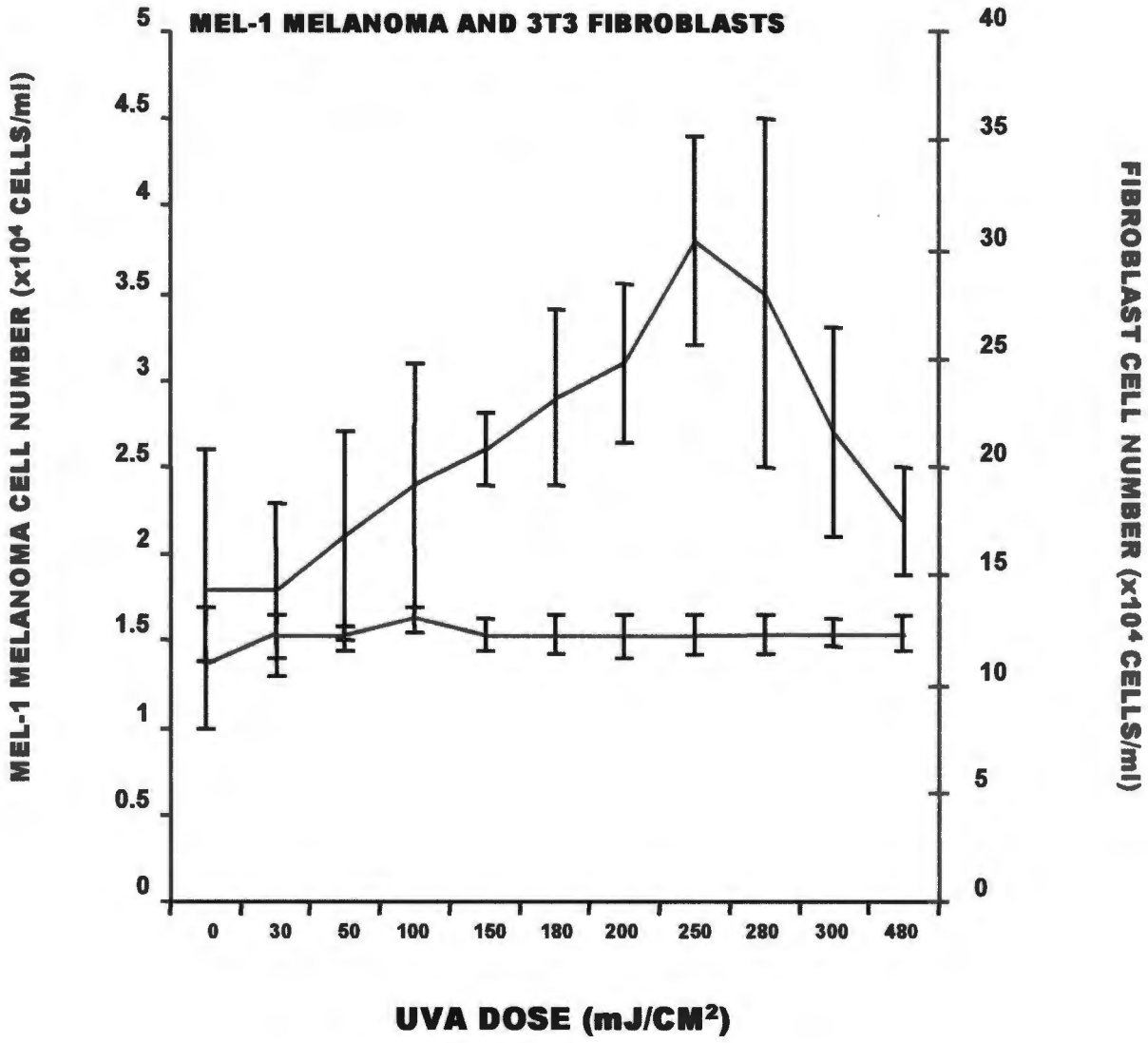


Figure 3.6: Effect of UVA on Proliferation of Mel-1 Melanoma cells and 3T3 Fibroblasts. Growth profiles of Mel-1 human melanoma cells (pink line) and 3T3 mouse fibroblasts (blue line), exposed to a single pulse of UVA light and then cultured for four days. The data depicted in this figure are presented as the mean number of cells ± S.E.M. of at least 6 separate experiments, each performed in triplicate.

was always used. When fibroblasts were exposed to the same range of UVA doses as the melanoma cells, proliferation was unaffected.

When the effect of UVA on the proliferation of NHMs was determined, only single dose experiments (using the optimal dose established for melanoma cells) were carried out (Figure 3.7). This was mainly due to the limited number of cells available for these experiments. In this study, a single dose of 250mJ/cm^2 UVA light had no effect on the proliferation of NHMs, compared to the sham-irradiated cells. However, in this figure it seemed as if UVA light had inhibited the proliferation of NHMs, but the t-test results revealed that differences were not significant at the $p < 0.05$ level, compared to the sham-irradiated control cells. This suggested that under the conditions of this experiment, UVA did not stimulate the proliferation of NHMs. These results were later confirmed by the results obtained in Figure 3.9. As before, the proliferation of 3T3 fibroblasts was not affected by UVA light.

Khellin and UVA (KUVA) treatment stimulates the proliferation of Mel-1 melanoma cells and NHMs, but inhibits the proliferation of fibroblasts.

The above results demonstrate that khellin alone and a single dose of UVA alone are able to stimulate the proliferation of melanoma cells. In NHMs, khellin alone but not UVA alone had a stimulatory proliferative effect. In order to determine the combined effect of these two treatments, cells were cultured overnight in 0.01mM or 0.1mM khellin, and then exposed to a single dose of 250mJ/cm^2 UVA. Following UVA exposure, the cells were cultured in khellin medium for a further four days. Results are presented in Figures 3.8, 3.9 and 3.10. In figures 3.8 and 3.10, a solid line represents the khellin and UVA combination (KUVA), while the dotted line represents the cells treated with khellin alone (unirradiated). The colour of each line represents a particular dose of khellin to which the cells were exposed.

The results clearly demonstrate that khellin plus UVA (KUVA) stimulates proliferation of melanoma cells more effectively than either khellin or UVA alone (Figure 3.8). Thus, for example, after four days in culture, 0.01mM khellin plus 250mJ/cm^{2}UVA (pink solid line) stimulated the proliferation 2.9-fold above the untreated controls (black dotted line), as compared to the 1.6-fold stimulation with 0.01mM khellin alone (pink dotted line) and the 1.2-fold stimulation with UVA alone (black solid line). This pattern of results was clearly visible even after the first day of KUVA treatment. Similarly, 0.1mM khellin plus UVA (green solid line) stimulated proliferation 1.8-fold,

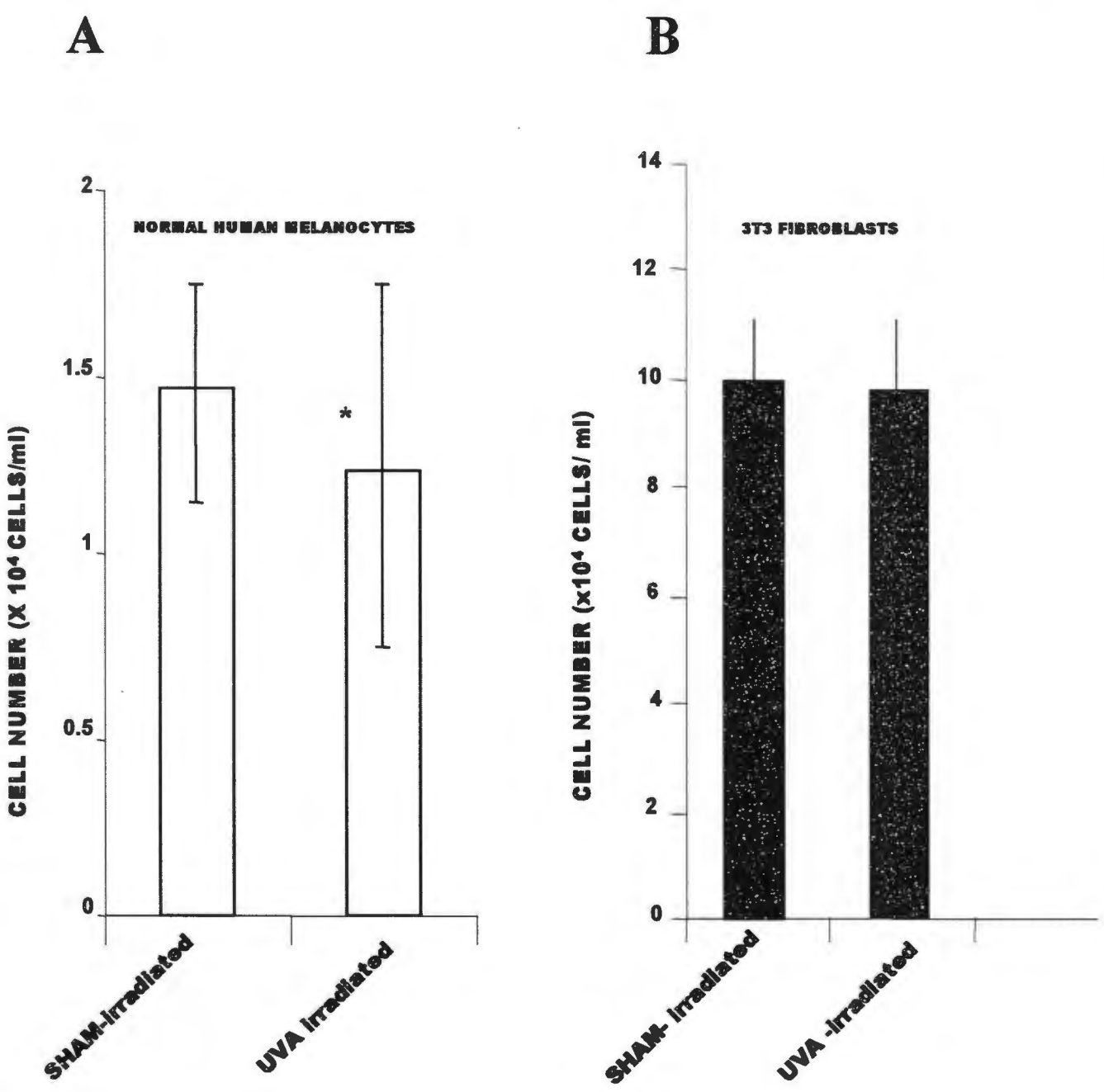


Figure 3.7: Effect of UVA on Proliferation of Normal Human Melanocytes (A) and 3T3 Mouse Fibroblasts (B). Growth profiles of normal human melanocytes (white columns) and 3T3 mouse fibroblasts (black columns) exposed to a single pulse of UVA light and cultured for four days. The sham-irradiated (control) cells were covered with black paper and exposed to the same experimental conditions as the irradiated cells. The data represented in this figure is the mean number of cells ± S.E.M of at least 5 separate experiments, each performed in triplicate. * $P > 0.05$ (result insignificant), versus sham-irradiated cells.

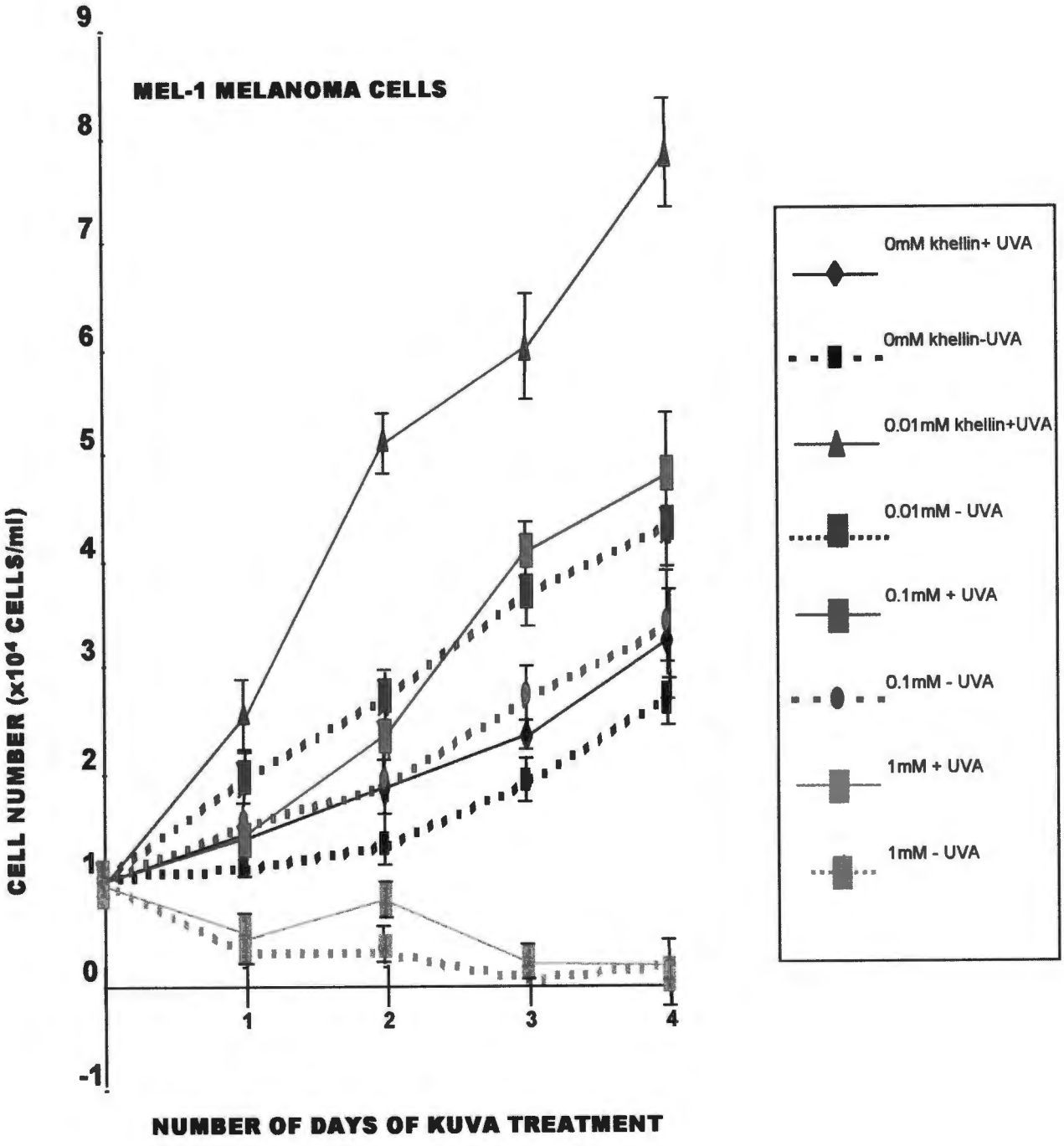


Figure 3.8: Effect of KUVA on Proliferation of Mel-1 Melanoma cells. Growth profile of Mel-1 melanoma cells grown in the presence of khellin and UVA (KUVA) over a four-day time period. Treatment of cells with the combination of khellin, 0.01mM (pink line) and 0.1mM (green line), and UVA is represented by a solid line, and without UVA (khellin alone), by a dotted line. The overall control is represented by a dotted black line (khellin and UVA absent). Data are represented as the mean cell number ± S.E.M. of at least 5 separate experiments, each performed in triplicate.

compared to the 1.3-fold stimulation with 0.1mM khellin alone (green dotted line) and the 1.2-fold stimulation with UVA alone (black solid line).

In order to determine whether the combination of 0.01mM khellin and UVA was acting additively or synergistically, the effect of individual and combined treatments on the population doublings was calculated, as described in Materials and Methods (Section 2.8). After four days, the untreated cells had undergone approximately 1.5 population doublings, whereas the cells treated with 0.01mM KUVA had undergone approximately 2.97 population doublings. Thus, the combined treatment (KUVA) increased the proliferation rate by 2.97-1.5 = 1.47 population doublings in 4 days, which is an increase of 98% (1.47 /1.5 x 100= 98%). In the same time period, cells treated with khellin alone, had undergone 2.1 population doublings. When compared to no treatment at all, this constituted an increase of 0.6 (2.1-1.5= 0.6) population doublings or 40% (0.6 / 1.5 x 100 = 40%). Treatment with UVA alone, however, resulted in 1.68 population doublings. This constituted an increase of 0.18 population doublings or an increase of 12% (0.18 / 1.5 x 100 = 12%). If the effects were additive, one would thus have expected an increase in the proliferation rate of 40% + 12% = 52% or less. However, both treatments combined led to almost double this increase in proliferation rate, namely 98%. This clearly indicates that the combination of khellin and UVA act synergistically to stimulate proliferation.

In order to determine whether the combination of 0.1mM khellin and UVA was acting additively or synergistically on the proliferation rate of the melanoma cells, the calculation as described above was also carried out. After four days of treatment, the untreated cells had undergone approximately 1.5 population doublings, whereas the cells treated with KUVA had undergone approximately 2.2 population doublings. Thus, the 0.1mM KUVA combination increased the proliferation rate by 2.2-1.5 = 0.7 population doublings in 4 days, which is an increase of 47%. In the same time period, cells treated with 0.1mM khellin alone had undergone 1.73 population doublings, which constituted an increase of 0.23 population doublings or an increase of 15.3%, when compared to no treatment at all. Treatment with UVA alone, however, resulted in 1.68 population doublings, which constituted an increase of 0.18 population doublings or an increase of 12%. If the effects were additive, one would thus have expected an increase in the proliferation rate of 15.3% + 12% = 27.3% or less. However, both treatments combined led to almost double this increase in proliferation rate, namely 47%. This also clearly indicates that the combination of 0.1mM khellin and UVA act synergistically to stimulate proliferation.

In this study, it was also determined whether the proliferation rate of NHMs would be affected by the combined treatment of 0.01mM khellin and UVA (see Figure 3.9). The results revealed that the 0.01mM KUVA combination stimulated the proliferation of these cells by 2-fold, in comparison to the 1.6-fold increase with 0.01mM khellin alone. UVA alone had no effect on the proliferation of NHMs, which confirmed previous findings. T-tests of NHMs treated with 0.01mM khellin and UVA (KUVA), revealed that the differences were significant at the $p < 0.05$ level, compared to the untreated control cells (untreated with both khellin and UVA).

As described for the melanoma cells, calculations were then carried out to determine whether the combination of 0.01mM khellin and UVA was acting additively or synergistically on the treated NHMs. These calculations may possibly be inaccurate due to the fact that an estimate of the starting cell number had to be made. It was estimated that after four days of treatment with 0.01mM KUVA, the cells had undergone 1.3 population doublings. Without any treatment, these cells had undergone 0.33 population doublings within the same time period. Thus, KUVA treatment increased the proliferation rate by 1.3-0.33= 0.97 population doubling in 4 days or 294% (0.97 / 0.33 x 100= 294%). In the same time period, cells treated with khellin alone had undergone 1 population doubling. When compared to no treatment at all, this constituted an increase of 0.67 (1-0.33= 0.67) population doublings or 203% (0.67 / 0.33 x 100 = 203%). Treatment with UVA alone, however, resulted in no increase in population doublings, compared to the untreated controls. If the effects of khellin and UVA were additive, one would thus have expected an increase in the proliferation rate of 203% + 0% = 203% or less. However, both treatments combined led to an increase in the proliferation rate of 294%. This clearly indicates that the combination of khellin and UVA results in a synergistic proliferative effect.

It was then calculated whether the combination of 0.1mM khellin and UVA was acting additively or synergistically on the proliferation rate of NHMs. After four days, the cells treated with KUVA had undergone 1 population doubling. Thus, the 0.1mM KUVA combination increased the proliferation rate by 1-0.33 = 0.67 population doublings in 4 days, which is an increase of 203%. In the same time period, cells treated with 0.1mM khellin alone had undergone 0.8 population doublings, which constituted an increase of 0.5 population doublings or an increase of 167%, when compared to no treatment at all. Treatment with UVA alone, however, resulted in no increase in population doublings, compared to the untreated cells. If the effects were additive, one would thus have expected an increase in the proliferation rate of 167% + 0% = 167% or less. However, both treatments combined led to an increase in the proliferation rate that was much more than this,

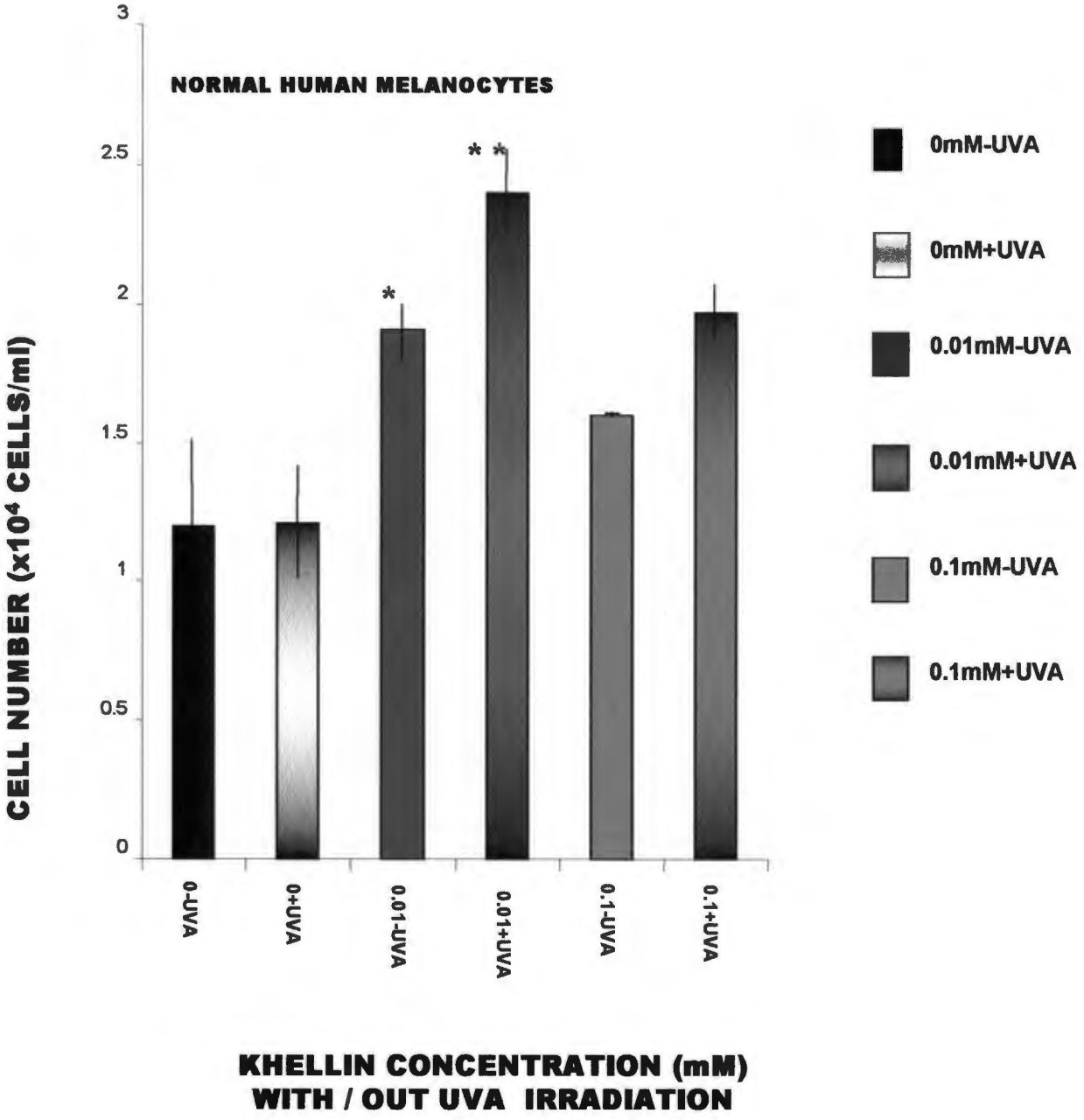


Figure 3.9: Effect of KUVA on Proliferation of Normal Human Melanocytes. Growth profiles of normal human melanocytes treated with khellin and UVA (KUVA). Treatment with khellin alone is represented by the single tone columns, 0.01mM (red columns) and 0.1mM (green columns), and dual-tone columns represent the combination of khellin and UVA (KUVA) treatments. The overall control (black column) of the study was only treated with medium and 1% DMSO. The cells treated with UVA alone are represented by the black dual-tone column. The data represented in this figure is the mean number of cells ± S.E.M of at least 3 separate experiments, each performed in triplicate. * $P < 0.05$ (significant) and ** $P < 0.01$ (result extremely significant), versus control.

namely 203%. This provides proof that the combination of 0.1mM khellin and UVA act synergistically to stimulate proliferation.

The effect of KUVA on 3T3 fibroblasts was also investigated, and the results shown in Figure 3.10, revealed that the proliferation of 3T3 fibroblasts was decreased with KUVA combination treatments. This growth inhibition was observed to be inversely proportional to the khellin doses. A very interesting finding was that the KUVA treatment was more cytotoxic to the fibroblasts than the treatment with khellin alone, at all concentrations.

In conclusion, KUVA combination treatments more effectively enhanced proliferation of melanoma cells and NHMs than khellin or UVA alone. The 0.01mM KUVA combination stimulated proliferation of both melanocytes and melanoma cells maximally. The proliferation of 3T3 fibroblasts was inhibited with all the KUVA combination treatments tested, and these were more cytotoxic than khellin or UVA alone.

Khellin, UVA and KUVA treatments enhance melanogenesis in human melanoma cells and NHMs.

To determine the effects of khellin alone, UVA alone and KUVA, on melanogenesis in Mel-1 cells and NHMs, the cells were treated as described for the proliferation assays, and their ability to synthesize melanin was measured using a standard radiometric assay. The results are presented as a percentage of the control activity. The percentage increase was obtained by subtracting the percentage control value from the percentage experimental value. For the assays with khellin alone, the cells were exposed to 0.01mM and 0.1mM khellin for four days as previously described. The protein was then harvested, and this was followed by the protein extracts being assayed. The results demonstrated that 0.01mM and 0.1mM khellin enhanced melanin formation in melanoma cells by 160% and 100%, respectively (Figure 3.11). T-tests revealed that the differences were significant at the $p < 0.05$ level, compared to the untreated control cells.

To determine the effect of UVA on the melanogenic activity of the melanoma cells, they were exposed to a single dose of 250mJ/cm^2 UVA light, cultured for four days and then the protein harvested for assaying. This treatment enhanced the melanogenic activity of Mel-1 melanoma cells by 30% above the sham-irradiated control cells (Figure 3.12). T-tests revealed that the differences were significant at the $p < 0.05$ level, compared to the sham-irradiated control cells.

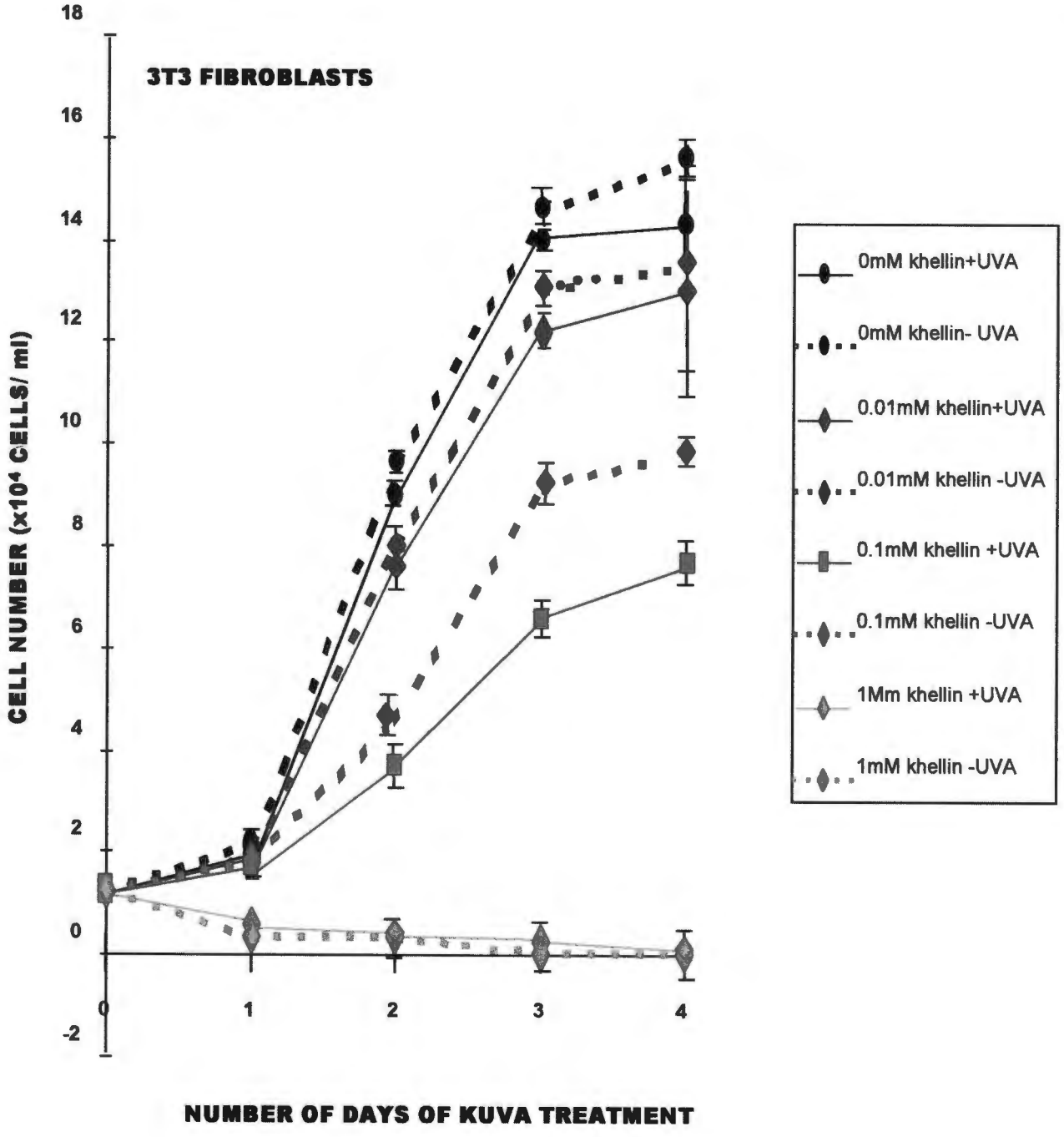


Figure 3.10: Effect of KUVA on Proliferation of 3T3 Fibroblasts. Growth profile of 3T3 mouse fibroblasts grown in the presence of khellin and UVA (KUVA) over a four-day time period. Treatment of cells with the combination of khellin, 0.01mM (pink line) and 0.1mM (green line), and UVA is represented by a solid line, and without UVA (khellin alone), by a dotted line. The overall control is represented by a dotted black line (khellin and UVA absent). Data are represented as the mean cell number ± S.E.M. of at least 5 separate experiments, each performed in triplicate.

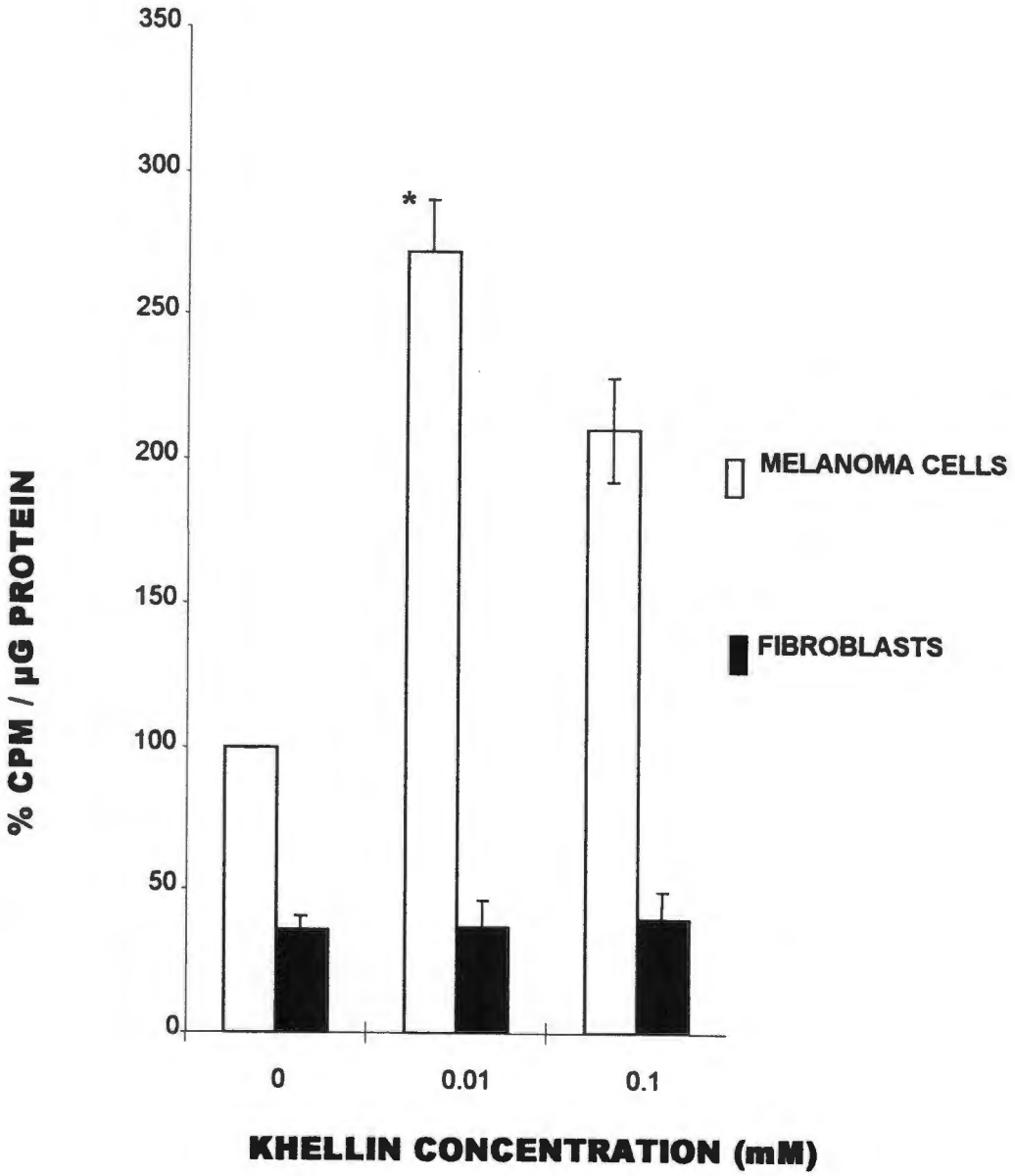


Figure 3.11 ^{14}C-Tyrosine Assay results of Mel-1 Melanoma cells and 3T3 Fibroblasts treated with Khellin. Human melanoma cells (white columns) and 3T3 mouse fibroblasts (black columns) were exposed to khellin for four days before protein was collected . Control cells were treated with medium alone and 1% DMSO. The data depicted in this figure are presented as the average percentage cpm/µg ± S.E.M. of at least 7 separate experiments, each performed in triplicate. The results are expressed as a percentage of the untreated control Mel-1 melanoma cells. * $P < 0.05$ (result significant), versus control.

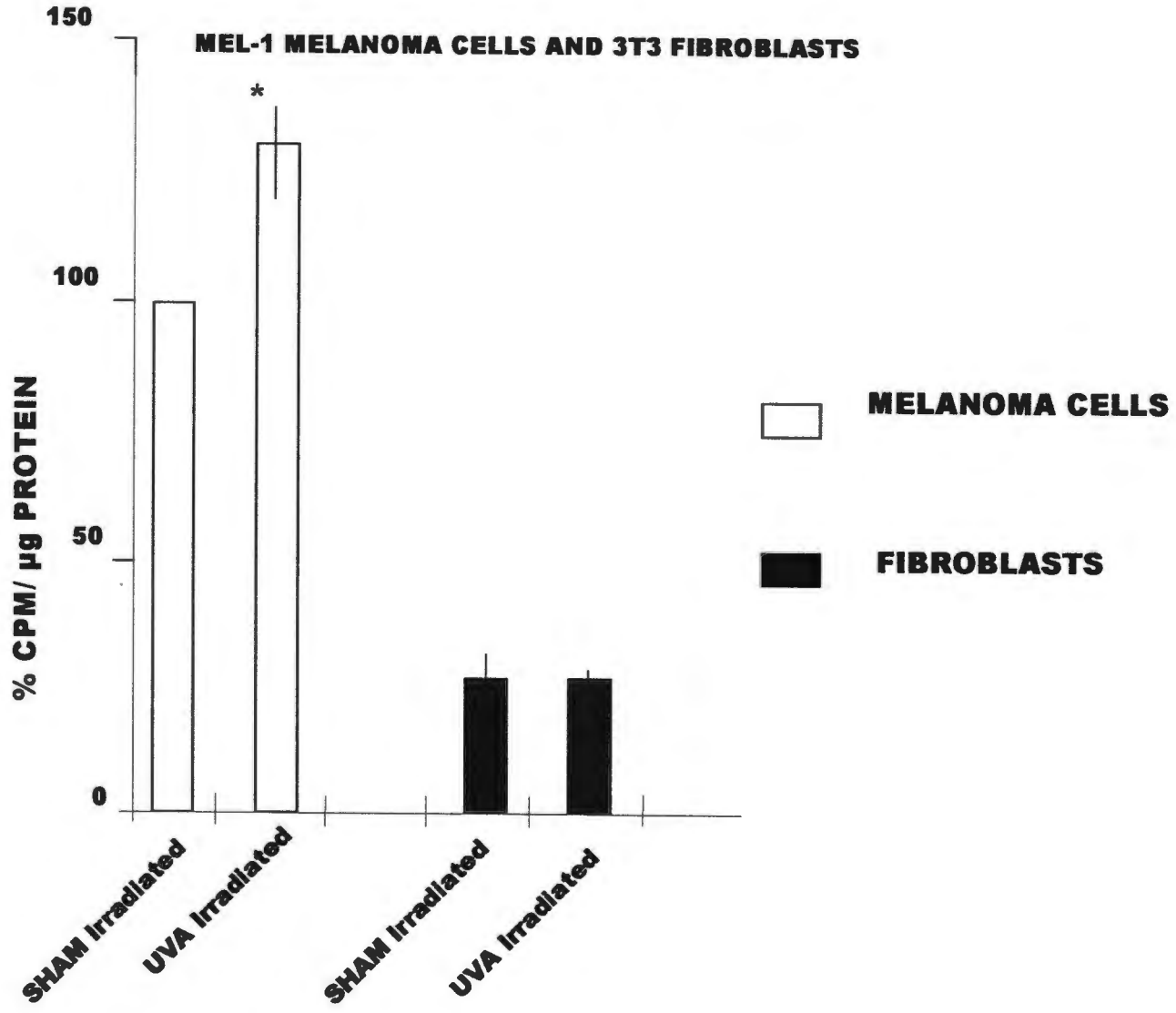


Figure 3.12 ^{14}C-Tyrosine Assay results of Mel-1 Melanoma cells and 3T3 Fibroblasts exposed to UVA light. Human Mel-1 melanoma cells (white columns) and 3T3 mouse fibroblasts (black columns) were exposed to a single dose of 250mJ/cm^2 UVA and cultured for four days before protein was collected . Control cells were covered with black paper in the UV cabinet. The data depicted in this figure are presented as the average percentage cpm/µg ± S.E.M. of at least 8 separate experiments, each performed in triplicate, as described in *Materials and Methods*. The results are expressed as a percentage of the unexposed control Mel-1 melanoma cells.* $P < 0.05$ (significant), versus sham-irradiated control

To determine the effect of combined KUVA treatments, the cells were cultured overnight in 0.01mM and 0.1mM khellin, exposed to a single dose of 250mJ/cm^2 UVA the following day, and then cultured for another four days in khellin before the protein was harvested for the assay. The results clearly demonstrated that khellin plus UVA (KUVA) stimulated the melanin formation of Mel-1 melanoma cells more effectively than either khellin, or UVA alone (Figure 3.13). Thus, for example, after four days in culture, 0.01mM khellin plus 250mJ/cm^2 UVA increased the melanin formation of melanoma cells by 200% above the untreated control cells, whereas the melanin formation was increased 100% by 0.01mM khellin alone and 30% by UVA alone. These results suggest that khellin in the presence of UVA, has a synergistic melanogenic effect on the melanoma cells in culture. This is because, if the effects of khellin and UVA were additive, one would thus have expected a 130% (100% + 30%= 130%) or less increase in the melanogenic activity of these cells. However, a 200% increase in the melanogenic activity of these cells had resulted, suggesting that the melanogenic effect of the combination of khellin and UVA is synergistic. With the 0.1mM KUVA combination, the melanogenic activity of these cells was increased by 130%, compared to the 80% increase with 0.1mM khellin alone and the 30% increase with UVA alone. This also clearly demonstrates that the 0.1mM KUVA combination treatment of melanoma cells increase their melanogenic activity synergistically. T-tests confirmed that the melanogenic assay results of Mel-1 cells treated with 0.01mM khellin and UVA, were significantly different at the $p < 0.05$ level, compared to the untreated control cells.

Like the melanoma cells, the melanogenesis of NHMs was increased in melanocytes treated with khellin alone (Figure 3.14). Exposure of NHMs to both 0.01mM and 0.1mM khellin increased the melanogenic activity of these cells by 154% and 100%, respectively. When the NHMs were exposed to a single dose of 250 mJ/cm^{2}UVA, the melanin formation rate was increased 30% above the sham-irradiated cells (Figure 3.15). T-tests revealed significant differences in the results that were obtained with 0.01mM khellin alone and UVA alone, at the $p < 0.05$ level, compared to the untreated control cells (Figures 3.14 and 3.15).

It was then determined whether the KUVA combination treatment had stimulated the melanogenesis in NHMs (Figure 3.16). The results revealed that after four days of treatment, 0.01mM khellin plus 250mJ/cm^2 UVA increased the melanin formation of NHMs by 290% above the untreated control cells, compared to the 130% increase with 0.01mM khellin alone and the 30% increase with UVA alone. These results suggest that khellin in the presence of UVA, has a synergistic melanogenic effect on NHMs in culture. This is because, if the effects of khellin and

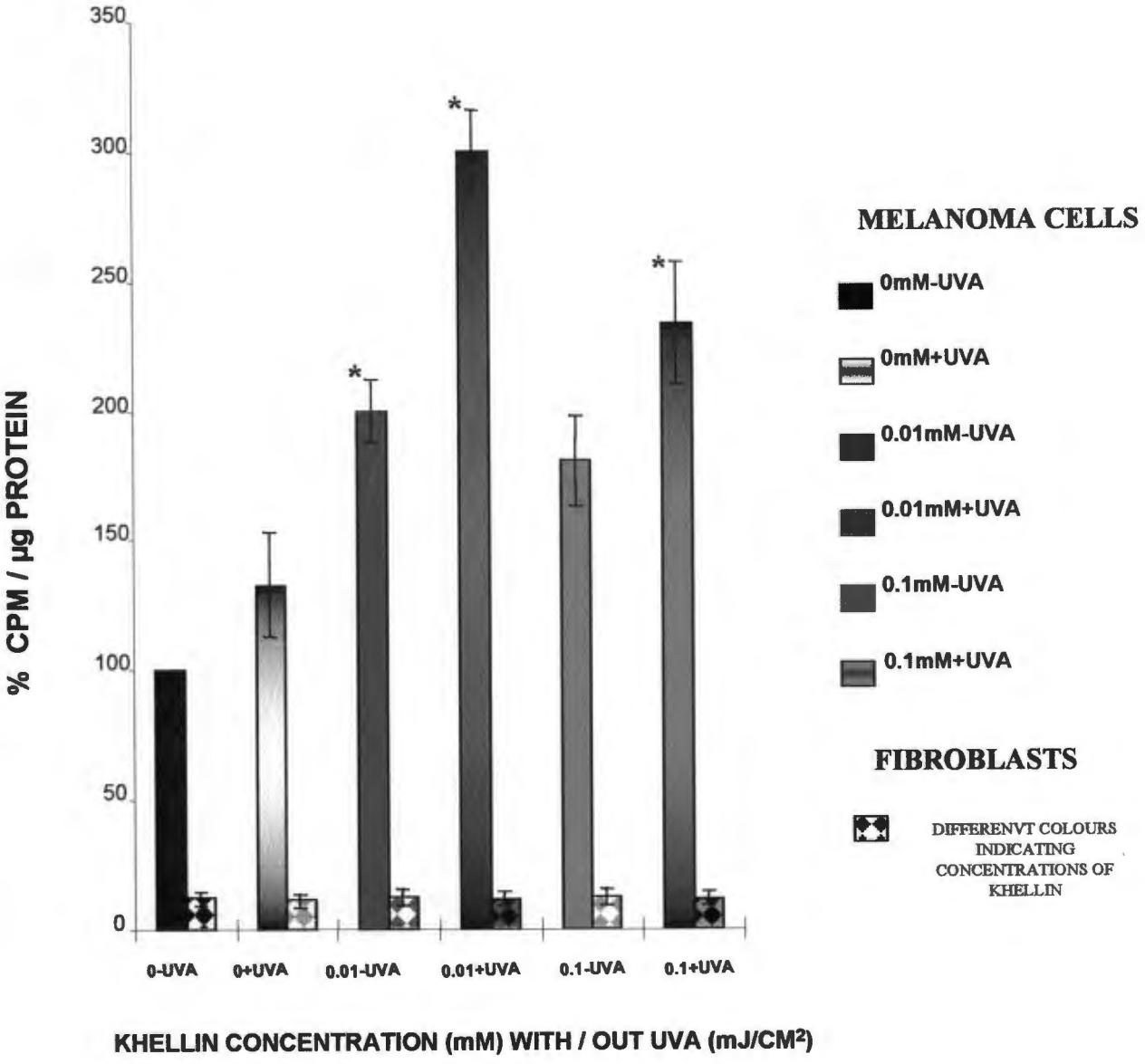


Figure 3.13: ^{14}C-Tyrosine assay results of Mel-1 Melanoma cells and 3T3 Fibroblasts treated with KUVA (khellin and UVA). Treatment with khellin alone is represented by the single tone columns, 0.01mM (red columns) and 0.1mM (green columns), and dual-tone columns represent the combination of khellin and UVA (KUVA) treatments. The overall control (black column) of the study was only treated with medium and 1% DMSO. Cells exposed to UVA alone are represented by the black dual-tone column. The data represented in this figure is the average percentage cpm/µg ± S.E.M. of at least 8 separate experiments, each performed in triplicate. The results are expressed as a percentage of the unexposed control Mel-1 melanoma cells. * $P < 0.05$ (result significant) versus control.

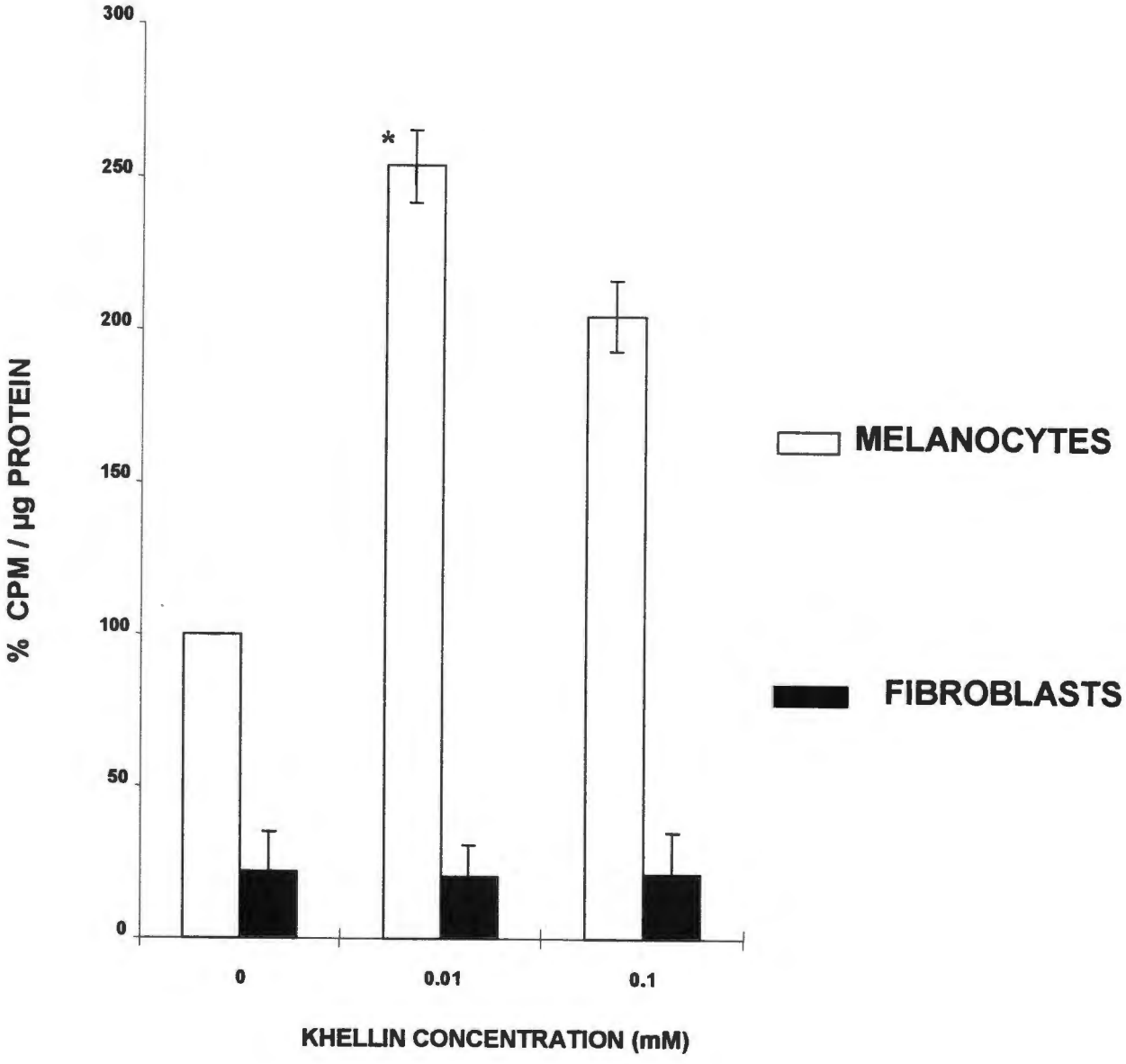


Figure 3.14 ^{14}C-Tyrosine Assay results of Normal Human Melanocytes and 3T3 Fibroblasts treated with Khellin. Normal melanocytes (white columns) and 3T3 mouse fibroblasts (black columns) were exposed to khellin for four days before protein was collected. Control cells were treated with medium alone and 1% DMSO. The data depicted in this figure are presented as the average percentage cpm/µg ± S.E.M. of at least 4 separate experiments, each performed in triplicate. The results are expressed as a percentage of the untreated control normal human melanocytes. * $P < 0.05$ (result significant), versus control

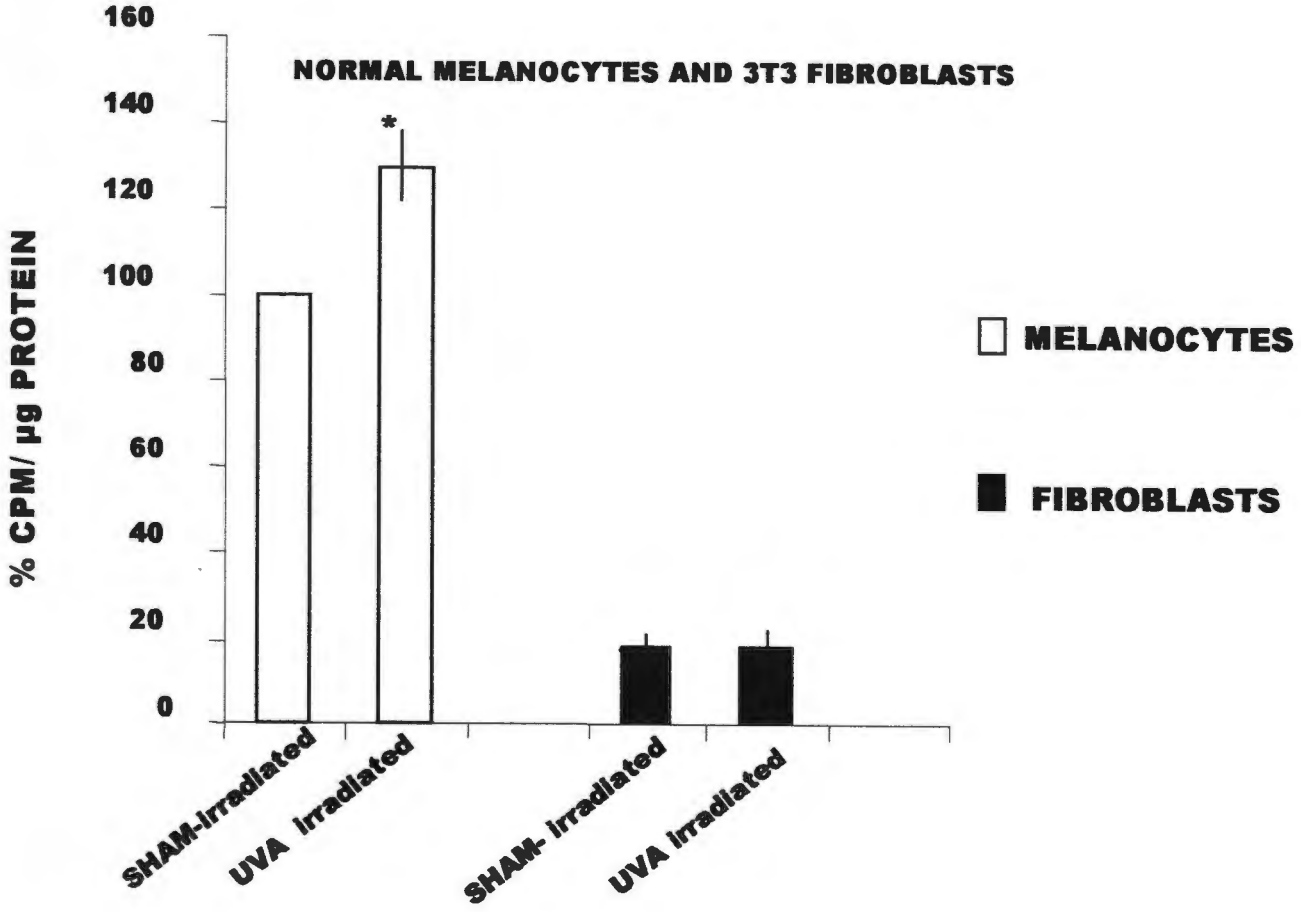


Figure 3.15 ^{14}C-Tyrosine Assay results of Normal Human Melanocytes and 3T3 Fibroblasts exposed to UVA light. Normal melanocytes (white columns) and 3T3 mouse fibroblasts (black columns) were exposed to a single dose of 250mJ/cm^2 UVA and cultured for four four days before protein was collected . Control cells were covered with black paper in the UV cabinet. The data depicted in this figure are presented as the average percentage cpm/µg ± S.E.M. of at least 8 separate experiments, each performed in triplicate. The results are expressed as a percentage of the unexposed control normal melanocytes. * $P < 0.05$ (result significant), versus control

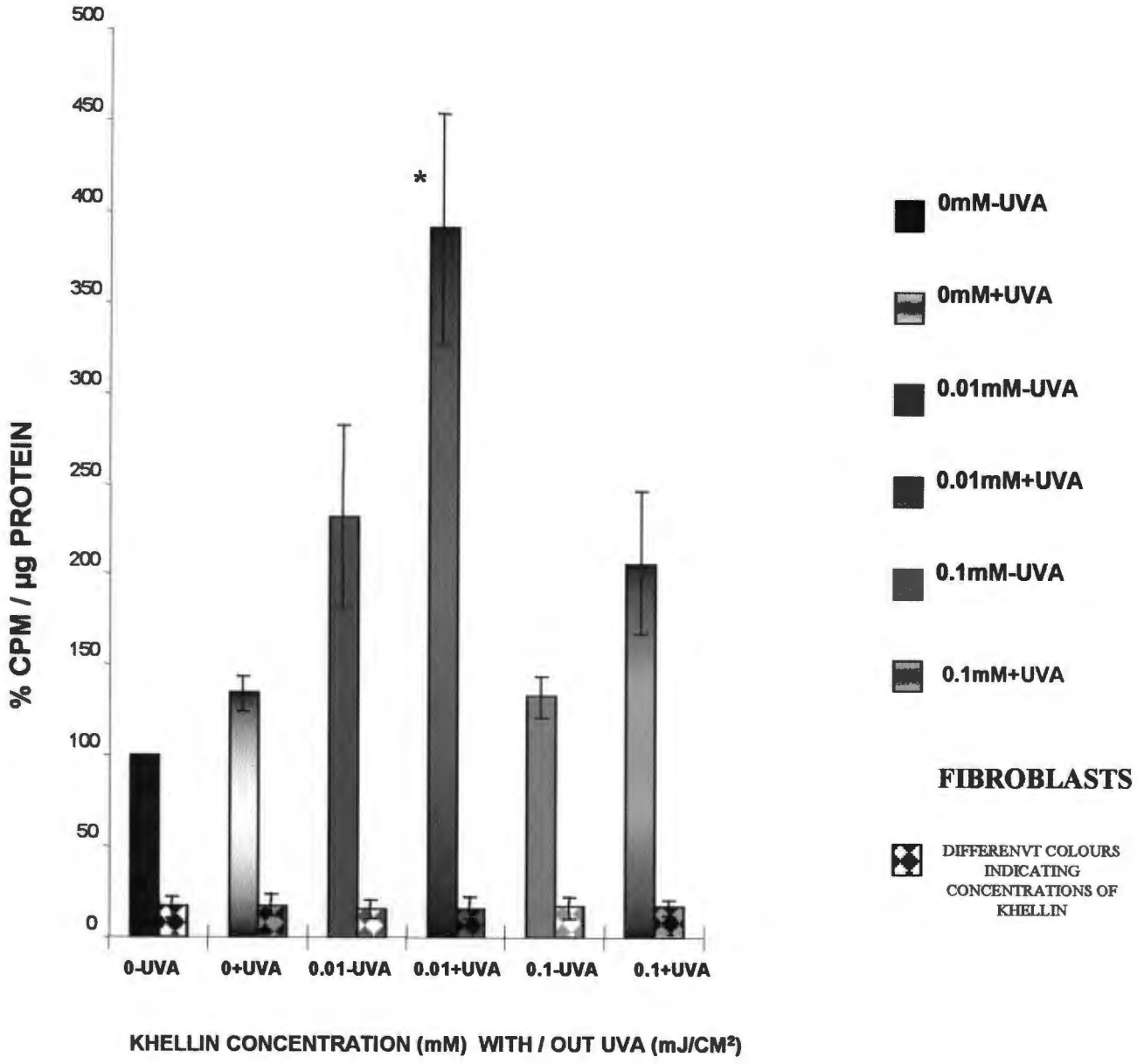


Figure 3.16: ^{14}C-Tyrosine assay results of Normal Human Melanocytes and 3T3 Fibroblasts treated with KUVA (khellin and UVA). Treatment with khellin alone is represented by the single tone columns, 0.01mM (red columns) and 0.1mM (green columns), and dual-tone columns represent the combination of khellin and UVA (KUVA) treatments. The overall control (black column) of the study was only treated with medium and 1% DMSO. Cells exposed to UVA alone are represented by the black dual-tone column. The data represented in this figure is the average percentage cpm/µg ± S.E.M. of at least 3 separate experiments, each performed in triplicate. The results are expressed as a percentage of the unexposed control normal melanocytes. * $P < 0.05$ (result significant), versus control.

UVA were additive, one would thus have expected an increase of 160% (130% + 30%= 160%) or less in the melanogenic activity of these cells. However, a 290% increase in the melanogenic activity of these cells had resulted, suggesting that the melanogenic effect of the combination of khellin and UVA is actually synergistic. Similarly, it was shown that the 0.1mM KUVA combination treatment enhanced melanin formation synergistically, since this treatment increased melanin formation by 110%, whereas both 0.1mM khellin alone and UVA alone increased melanin formation by 30% each. T-tests revealed significant differences in the results that were obtained with 0.01mM khellin plus UVA at the $p < 0.05$ level, compared to the untreated control cells.

As expected, treatment of 3T3 fibroblasts with khellin alone, UVA alone and KUVA had no effect on the melanin formation, compared to the untreated cells (Figures 3.14, 3.15 and 3.16). The low level of ^{14}C-tyrosine incorporation seen in the 3T3 fibroblasts represents background values.

In conclusion, these results clearly indicate that khellin and even more so KUVA, enhances the melanogenic activity of both NHMs and Mel-1 melanoma cells *in vitro*. Interestingly, these results also suggest that the maximal proliferative dose of khellin is also the maximal melanogenic dose.

Levels of melanogenic enzymes increased in KUVA-treated Mel-1 melanoma cells and NHMs.

To determine whether the increase in melanogenic activity in KUVA-treated melanocytic cells was due to an increase in the levels of melanogenic enzymes, western blot analyses of the KUVA-treated cells (melanoma and normal human melanocytes) and their controls were carried out. The cells were exposed to khellin and UVA as described earlier. The antibodies used in this study included α-PEP-8, (a rabbit polyclonal antibody against the carboxy terminus of mouse dopachrome tautomerase, TRP-2), α-PEP-7 (a rabbit polyclonal antibody against the carboxy terminus of mouse tyrosinase) and TMH-1 (a rat monoclonal antibody which recognizes mouse TRP-1). All of these antibodies cross-react with human antigens, with lower affinity than to murine antigens (V. Hearing, personal communication). For each western blot experiment, B16 mouse melanoma protein extract was included as the positive control and protein extract from 3T3 fibroblasts was included as a negative control.

Immunoblotting analyses of TRP 2 protein of melanoma cells treated with khellin alone or in combination with UVA were first carried out. The results revealed the presence of two bands of about 67kDa and 75kDa (see arrows, Figure 3.17) in all the lanes, including the positive control

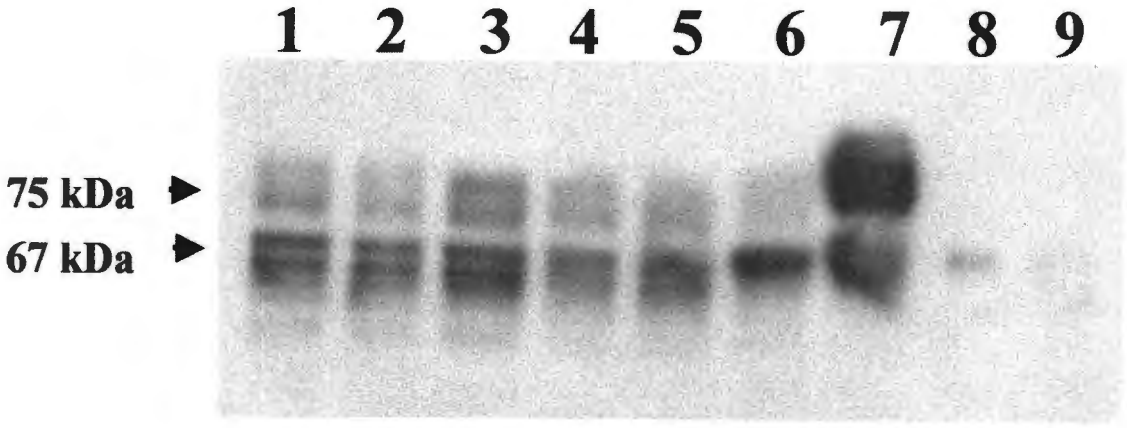


Figure 3.17: Western immunoblot analysis of levels of TRP-2 (α-PEP 8) melanogenic protein of MEL-1 MELANOMA CELLS treated with KUVA, khellin alone and UVA alone. The cells were solubilized in lysis buffer and 40μg protein from each extract was separated on a 7.5% SDS gel. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to TRP-2 (α-PEP-8), and the bands visualized by chemiluminescence. Cells were treated as follows:

LANE 1: Melanoma cells treated with 0mM khellin + UVA
LANE2: Melanoma cells treated with 0mM khellin - UVA
LANE3: Melanoma cells treated with 0.01Mm khellin + UVA
LANE4: Melanoma cells treated with 0.01mM khellin - UVA
LANE5: Melanoma cells treated with 0.1mM khellin + UVA
LANE6: Melanoma cells treated with 0.1mM khellin - UVA

LANE7: B16 melanoma cell extract

LANE8: 3T3 fibroblasts treated with 0.01mM khellin + UVA

LANE9: 3T3 fibroblasts treated with 0.01mM khellin - UVA

cells (lane 7). In each lane, the bands consistently appeared as a doublet in repeat assays. In contrast, western blots of normal human melanocytes with α-PEP 8 (α-TRP 2) consistently revealed two single bands (see Figure 3.18). The appearance of doublets in melanoma cells is possibly due to alterations, mutations or alternative splicing of the melanogenic enzymes in this cell line. It is assumed that the upper doublet or band is the glycosylated form of TRP 2 and the lower doublet or band is the non-glycosylated form. A faint non-glycosylated band was present in the cell extract of 3T3 fibroblasts (lane 8). This was possibly due to either a non-specific cross-reaction to the antibody, or alternatively it represented the TRP-2 expression by fibroblasts (as reported by Tachibana et. al., 1996,).

As seen in Figure 3.17, the highest level of TRP-2 protein was observed in the lanes of the melanoma cell extracts treated with KUVA, as compared to the controls, which were untreated (lane 2) or treated with khellin alone (lanes 4 and 6). The band present in cells treated with the 0.01mM KUVA combination was the most intense on the blot. It was also found that in all samples treated with UVA (lanes 1, 3, 5) an increase in the glycosylated form of the TRP-2 protein was obtained. In addition, in the presence of UVA, both 0.01mM and 0.1mM khellin increased *de novo* synthesis of the TRP-2 melanogenic protein. When comparing the bands of the untreated cells to that treated with khellin alone, it would seem that khellin alone had no effect on glycosylation of TRP-2.

As with melanoma cells, immunoblotting analyses of KUVA-treated NHMs revealed both glycosylated and non-glycosylated forms of TRP-2 in all the lanes of the melanocytes and in the B16 melanoma control (Figure 3.18). It was also found that KUVA treatments resulted in an increase in the glycosylated form of TRP-2, with the 0.01mM KUVA combination being the most effective. In comparison to the control cells, khellin alone (0.01mM and 0.1mM) had no effect, and a marginal increase in the glycosylated form of the protein was obtained with UVA alone. As seen before, the fibroblasts displayed a faint non-glycosylated band on the blots.

Despite numerous attempts, tyrosinase could not be detected in the Mel-1 melanoma cells. This suggests that this melanoma cell line as with many others, contain very low levels of tyrosinase. Tyrosinase was however, detected in NHMs and it was visible as two bands of about 65kDa and 70kDa (see arrows, Figure 3.19). The upper band represents the glycosylated form and the faint lower band represented the *de novo* form of the protein. Examination of this figure revealed that the glycosylated form was more prevalent in the KUVA-treated samples than the *de novo* form of the

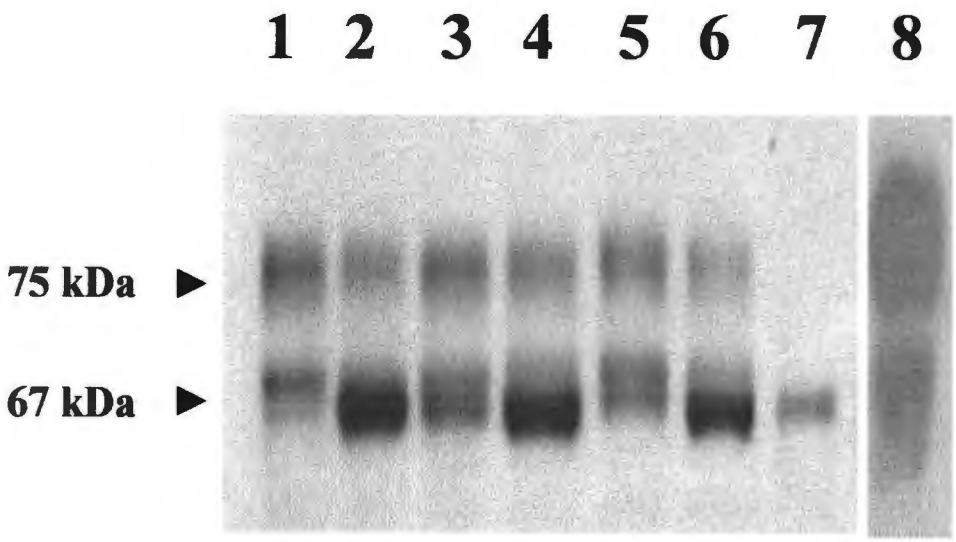


Figure 3.18: Western immunoblot analysis of levels of TRP-2 (α-PEP 8) melanogenic protein of NORMAL HUMAN MELANOCYTES treated with KUVA, khellin alone and UVA alone. The cells were solubilized in lysis buffer and 40μg protein from each extract was separated on a 7.5% SDS gel. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to TRP-2 (α-PEP-8), and the bands visualized by chemiluminescence. Cells were treated as follows:

LANE 1: Normal melanocytes treated with 0mM khellin + UVA
LANE2: Normal melanocytes treated with 0mM khellin - UVA
LANE3: Normal melanocytes treated with 0.01Mm khellin + UVA
LANE4: Normal melanocytes treated with 0.01mM khellin - UVA
LANE5: Normal melanocytes treated with 0.1mM khellin + UVA
LANE6: Normal melanocytes treated with 0.1mM khellin - UVA

LANE7: 3T3 fibroblasts treated with 0.01mM khellin + UVA

LANE8: B16 melanoma cell extract

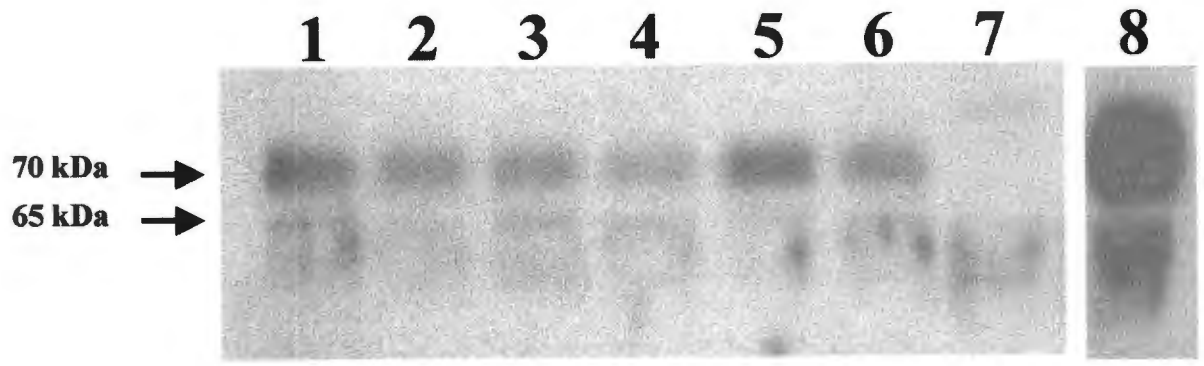


Figure 3.19: Western immunoblot analysis of levels of TYROSINASE (α-PEP7) melanogenic protein of NORMAL HUMAN MELANOCYTES treated with KUVA, khellin alone and UVA alone. The cells were solubilized in lysis buffer and 40μg protein from each extract was separated on a 7.5% SDS gel. After electrotransfer to nitrocellulose membranes, the blots were probed with antisera specific to tyrosinase (α-PEP-7), and the bands visualized by chemiluminescence. Cells were treated as follows:

LANE 1: Normal melanocytes treated with 0mM khellin + UVA
LANE2: Normal melanocytes treated with 0mM khellin - UVA
LANE3: Normal melanocytes treated with 0.01Mm khellin + UVA
LANE4: Normal melanocytes treated with 0.01mM khellin - UVA
LANE5: Normal melanocytes treated with 0.1mM khellin + UVA
LANE6: Normal melanocytes treated with 0.1mM khellin - UVA

LANE7: 3T3 fibroblasts treated with 0.01mM khellin + UVA

LANE8: B16 melanoma cell extract

protein. The glycosylated form was more prevalent in all UVA-treated samples. Since khellin alone had no effect on tyrosinase protein levels, and the band intensities of the KUVA-treated samples were not any different from the samples treated with UVA alone, no absolute conclusions could be made about the tyrosinase protein levels in the KUVA-treated melanocytes.

In this study, immunodetection of the TRP-1 melanogenic protein in both untreated and KUVA-treated melanoma cells and NHMs was also carried out. Despite numerous attempts and procedural modifications, this protein could not be detected in both the Mel-1 melanoma cells and B16 melanoma controls. The inability to immunodetect TRP-1 was unlikely to be due to the cell extracts not expressing this enzyme, but more likely to be a technical problem.

In summary, TRP-2 protein levels were increased most evidently in cultured melanoma cells and normal human melanocytes treated with both khellin and UVA. It was found that the 0.01mM KUVA combination was the most effective treatment in increasing TRP-2 protein levels. KUVA treatment of the melanoma cells increased both glycosylation and *de novo* synthesis of the TRP-2 protein, whereas in NHMs, an increase in glycosylation of the protein was only obtained. However, khellin alone had no effect on the TRP-2 protein levels in both cultured melanocytes and melanoma cells, whereas UVA alone had marginally increased the levels in these cells. The western blot result of tyrosinase protein levels in KUVA-treated melanocytes was not entirely clear. Since these antibodies are more appropriate for use on mouse samples, as they are targeted to recognize the mouse gene products rather than the human gene products, a reduction in sensitivity of detection may have occurred. Thus the conclusion was drawn that no tyrosinase was detectable in the melanoma cells. However, from these results, it would appear that UVA was responsible for the increase in glycosylation of tyrosinase in the KUVA-treated melanocytes. It should be noted that this immunodetection analyses was only done once, and experimental errors could possibly have occurred.

CHAPTER 4

DISCUSSION:

KUVA photochemotherapy seems to be a promising new treatment for repigmenting vitiliginous skin. KUVA is reported to be as effective as PUVA photochemotherapy in stimulating repigmentation, but without its side effects (Abdel-Fattah, 1982; Honigsman et. al., 1985; Ortel et. al., 1988; Orrechia and Perfetti, 1992), although the efficacy of khellin remains in dispute (Honigsman et. al., 1985; Ortel et. al., 1988; Procaccini et. al., 1995; Milne et. al., 1999, manuscript submitted). The mechanism by which khellin stimulates repigmentation is not yet known, nor is it known whether khellin acts directly on the melanocytes, nor whether it acts in combination with surrounding tissues and cells. What is of particular concern, is the fact that this drug is being used in clinical trials (Milne et. al., 1999), even though it is not known whether khellin has a cytotoxic dose range at all. If a cytotoxic dose is delivered to the skin cells during treatment, an accelerated loss of the already scarce melanocytes in the skin and hair of vitiligo patients may occur, resulting in even further skin depigmentation.

This study was carried out in order to shed some light on the mechanism of KUVA photochemotherapy. The main aim of this study was to use a cell biological system to investigate whether melanocytic cells are directly affected by treatment with khellin alone and in combination with UVA (KUVA). This involved determining if these treatments affected the processes of proliferation and melanogenesis of melanoma cells and normal human melanocytes.

Khellin and KUVA stimulate proliferation of melanocytes and melanoma cells

To determine whether khellin has a direct effect on the proliferation of melanocytic and non-melanocytic cells in culture, the present study was initiated by carrying out dose-concentration analyses of four-day cultures of melanoma cells treated with khellin. The results demonstrated that khellin stimulated the proliferation of melanoma cells over a wide concentration range, with a maximal stimulation at 0.01mM. Similar results were obtained with cultures of NHMs. Khellin inhibited the proliferation of fibroblasts at all concentrations tested. Treatment with UVA alone stimulated the proliferation of melanoma cells, but not NHMs. This is similar to the results of Kao and Yu, (1992), though different doses of UVA were used.

The most significant finding of the proliferation studies was that lower doses of khellin (0.01mM) combined with a single dose (250mJ/cm^2) UVA resulted in a synergistic stimulation of melanocyte and melanoma cell proliferation. These results provide strong support for the clinical studies, which indicate that KUVA treatment stimulates melanocyte proliferation, and further indicate that KUVA acts directly on the melanocytes to bring about this effect. This does not exclude the possibility that KUVA might also be acting in a paracrine fashion *in vivo*. These results also clearly underline the importance of combining khellin with UVA (KUVA) as a novel therapy for the effective treatment of vitiligo.

How does khellin and KUVA stimulate melanocyte proliferation?

There are a number of possible mechanisms whereby khellin and KUVA could stimulate melanocyte proliferation. The first possibility is that khellin alone, UVA alone, or the photochemically modified form of khellin, interact with cell membrane lipids and cause the release of diacylglycerols (DAGs). Previous investigators have reported that in the presence of UVA light, psoralen, which is structurally similar to khellin, interacts with membrane lipids to form psoralen-fatty acid adducts (reviewed Zarebska et. al., 1994; Anthony et. al., 1997; Frank et. al., 1998), which are reported to be structurally similar to DAGs (Frank et. al., 1994; Caffieri et. al., 1996; Anthony et. al., 1997). Since tumour-promoter esters such as TPA, are reported to have a DAG-like structure (Castagna et. al., 1982; Nishizuka, 1984; 1988), it is therefore possible that KUVA increases melanocytic cell proliferation by mimicking the action of these tumour promoters.

A second explanation for the increase in melanocyte proliferation (and melanogenesis) with KUVA photochemotherapy is that KUVA possibly mimics the down-stream events of ET-1 signalling (See Figure 1.5). ET-1 has been shown by others to stimulate both melanocyte proliferation and melanogenesis concomitantly, by activating the PKC, PKA and MAP kinase signal transduction pathways (Yada et. al., 1991; Imokawa et. al., 1996; 1997). Supporting this theory was the study that showed PKA-elevating agents such as cAMP increase both melanocyte proliferation and melanogenesis (Bertolotto et. al., 1997). In addition, activation of the PKC pathway initiates a cascade of events that lead to the phosphorylation of a pivotal protein kinase, MAP kinase, which in turn phosphorylates and activates nuclear transcription factors, which are involved in both proliferation and melanogenesis of the cell. Furthermore, Shoji et. al. (1998), demonstrated that melanocytes were five times more responsive to PKC-activating agents than the non-melanocytic

keratinocytes. Therefore, if KUVA is a PKC-activating agent, it would explain why the melanocytes in this study are more responsive to KUVA treatment than the non-melanocytic fibroblasts. Moreover, a study by di Stefano et. al. (1996), provided evidence that khellin initiates a cascade of intracellular responses by interacting with a pertussis toxin-sensitive Gi receptor protein. The ET-1 receptor is a Gi coupled receptor (Imokawa et. al., 1997, Figure 1.5). It is therefore possible that khellin and KUVA act via the same receptor and along the same signal transduction pathway that ET-1 acts, and that this is responsible for the repigmentation of vitiligo skin.

A third possible explanation for the increase in cell number is that KUVA-induced cell stress (such as DNA crosslinking or photo-adduct formation) may lead to the activation of transcription factors involved in the processes of proliferation such as AP-1 (activational protein-1, Gasparro et. al., 1997). Previous investigators have shown that khellin alone and even more so KUVA formed DNA crosslinks *in vitro* (Vedaldi et. al., 1988; Morliere et. al., 1988; Riccio et. al., 1992). This may thus result in DNA damage or cell stress. Gasparro et. al. (1997) suggest that during the repair of the cell, DNA becomes unwound in order to restore the correct nucleotide sequence, and cellular transcription factors could then gain access to sites previously occluded by the native chromosome structure. Thus, transcription factors involved in the proliferation process could then possibly gain access to the nuclear area of the cell.

The last possible explanation for the increase in cell numbers after khellin or KUVA treatment is that a prolongation of the G2 phase of the cell cycle might have occurred. Prolongation of the G2 phase of the cell cycle is known to be a generalized cellular response to injury (Bolognia et. al., 1994). Cellular injury may have occurred when DNA crosslinks are formed within the cells treated with khellin or KUVA. The G2 phase is the phase during which MSH is most active and this may result, in an increase in cell proliferation (and melanogenesis) in melanoma cells and normal human melanocytes (Hedley et. al., 1998). However, in the present study, the culture medium was not supplemented with MSH, suggesting either that the hypothesis is not valid or some other mechanism is also operational. To test this hypothesis one could culture cells in the presence or absence of KUVA and test their responsiveness to MSH. Another possibility is that one could measure whether cells increase their number of MSH receptors in response to KUVA treatment.

Why do normal melanocytes and melanoma cells respond to UVA light differently?

In the present study, the NHMs were not stimulated to proliferate in the presence of UVA light,

whereas melanoma cells did. At present, there is no agreement about how UVA light affects the proliferation of cultured melanocytic cells, because of different results obtained by different groups (Willis et. al., 1972; Blog et. al., 1979; Kilgman and Kligman, 1985; Rosen et. al., 1987; Ortonne, 1990; Kao and Yu, 1992; Mengeaud and Ortonne, 1996). It is likely that the differences in the results are due to differences in experimental designs (single versus multiple exposures), culture conditions, different UVA doses or spectral outputs of the light sources used. One possibility not considered by others is that the different responses relate to different levels of melanization. The Mel-1 melanoma cells are less pigmented and have lower levels of melanogenic activity as demonstrated by the lower incorporation values of the melanoma cells in the melanin formation assays (see Appendix, and also see Figure 2.1). It is known that the higher levels of melanin in normal human melanocytes protect the cells from DNA damage by quenching free radicals (Kobayashi et. al., 1993; Memoli et. al., 1997; Im et. al., 1998). The consequence of this is that there would be less DNA damage in the pigmented melanocytes and therefore critical cellular processes of the cells, such as proliferation, would not be stimulated. This proposal seems plausible since UVA light is said to bring about its biological effects by causing oxidative DNA damage by the release of reactive oxygen species (ROS) (Applegate and Frenk, 1995; Gilchrest et. al., 1996). Thus, the melanin present in the NHMs may protect the cells against damage to the DNA or cell membranes, and lower the chance of biological processes of the cell being disrupted.

Another possible explanation for the failure to observe enhanced proliferation of normal human melanocytes in the presence of UVA is that the melanocytes are slow growing and under the conditions of the present experiments, small differences in proliferation might not have been detected. Future studies could include longer culture periods together with multiple UVA treatments.

Cytotoxicity of khellin

In the present study, it was found that khellin was cytotoxic to both melanocytic cells (melanoma cells and normal human melanocytes) and non-melanocytic cells (fibroblasts) at concentrations above 0.5mM. The degree of cytotoxicty was found to rise with increasing concentrations of khellin. The cytotoxic assays revealed that 1mM khellin was equally cytotoxic to both melanoma cells and fibroblasts. It is likely that the cytotoxicity is due to the formation of DNA crosslinks, which leads to DNA damage, failure to repair and ultimately cell death.

The results of the present study also indicate that at certain concentrations (10^{-9} –10^{-4} M) khellin stimulates the proliferation of the melanocytes, but inhibits fibroblast proliferation. Trypan-blue dye exclusion assays suggest that at lower concentrations the khellin is merely cytostatic, as there is little evidence of cell death at these concentrations. Furthermore, it was found that the KUVA combination treatments were more cytotoxic to the fibroblasts than khellin alone. This suggests that a new photochemical product is possibly formed when khellin and UVA are combined. This suggestion awaits formal testing.

It is not clear why KUVA treatments are more toxic to fibroblasts than melanocytes. It is possible that fibroblasts are more sensitive to DNA-damaging agents than the melanocytic cells, because melanin and its intermediate products protect the pigmented melanocytic cells from damage by scavenging free radicals (Schmitz et. al., 1995; Memoli et. al., 1997; Prota, 1997). Damage may result from KUVA-induced DNA crosslinks and from the free radicals produced during UVA exposure, as described earlier. Because fibroblasts do not contain melanin, they would be more sensitive to lower doses of KUVA treatment. Alternatively, it is possible that the difference in the proliferative response of fibroblasts and melanocytic cells is due to a limitation imposed by the experimental design: Within the four days of analysis, the faster-growing fibroblasts would have undergone more population doublings than the slower-growing melanoma cells. Therefore, the effect of khellin on the proliferation might have been more evident because one is in fact evaluating a larger number of cells (i.e. measuring the effect on increased population doublings). For both melanoma cells and fibroblasts to have undergone equivalent population doublings, longer-term cultures of melanoma cells would be needed. To test this theory, long-term cultures or the use of cell lines with similar growth rates should be included in future experimental protocols.

The fibroblast cell line used in the study was an immortal murine cell line, and the results may have been different if a human fibroblast cell line was used. It is possible that this mouse cell line is more sensitive to damage by this drug than its human counterpart. An explanation for the failure of mouse fibroblasts to proliferate in response to khellin is possibly because in murine cells, no or markedly low rates of excision repair mechanisms take place after DNA damage (Vijg et. al., 1984; Applegate and Ley, 1996; Rosenstein and Rosenstein, 1992), as compared to human cells. Because of the absence of excision repair mechanisms in the fibroblasts, the damage to the cells is not removed and therefore growth inhibition possibly occurs. Alternatively, lack of repair in the cells may possibly result in the tumour-suppressor genes, for example p53, being up-regulated and thus preventing the cells from dividing, as was shown by Maltzman et. al. (1984).

The effect of PUVA versus KUVA on melanocyte proliferation *in vitro*

When the results of the present study were compared to equivalent studies on the effect of PUVA on the proliferation of melanocytes (Kao and Yu, 1992; Mengeaud and Ortonne, 1996; Luftl et. al., 1998), it was found that the KUVA results were different to those obtained with PUVA. It was found that whereas KUVA stimulated proliferation, PUVA inhibited proliferation. The likely explanation for the inhibition of the proliferation rate of cultured melanocytic cells with PUVA is that it is that PUVA is known to be more genotoxic than KUVA *in vitro,* because khellin forms fewer DNA crosslinks than psoralen *in vitro*. Since DNA crosslinking is likely to inhibit DNA replication, it is possible that PUVA is more growth inhibitory than KUVA. It is important to note that in the proliferation study with PUVA of Kao and Yu, (1992), high concentrations of psoralen was used and it is quite possible that at lower concentrations the drug would not have been as inhibitory. The results of the present study thus demonstrate the importance of testing drugs over a wide range of concentrations.

Khellin and UVA act synergistically to stimulate melanogenesis in melanocytes and melanoma cells

The results of the present study show that UVA alone and khellin alone stimulate melanogenesis in NHMs and melanoma cells. Significantly, the results further show that when added together there is a synergistic increase in melanogenesis. This synergism is most evident when the melanocytic cells are treated with 0.01mM khellin plus a single dose of 250mJ/cm^2 UVA. At this dosage, melanogenesis was increased by 200% and 290% in melanoma cells and NHMs respectively. Multiple doses of UVA were not tested in this study.

In order to explore the molecular mechanisms by which khellin and UVA bring about this synergistic response, the first question was to determine whether the response was due to an increase in the synthesis of melanocyte-specific enzymes or a post-translational activation of these enzymes. If the response was due to new enzyme synthesis, one might expect to detect an increase in the total amount of protein in khellin and KUVA treated cells. However, the results of the western blots do not support this theory and there was little or no significant increase in the total amount of tyrosinase or TRP-2 in khellin- and KUVA-treated cells. These results strongly suggest that under the conditions of these experiments, khellin and even more so KUVA, bring about an

increase in melanogenesis by post-translational enzyme activation or by the inactivation and/or removal of some melanogenic inhibitor (Kameyama, 1993, see Figure 4.1)

There are a number of theories that could be put forward to explain these results. As described in the Introduction, UV light and psoralens have been shown to interact with membrane lipids, resulting in the release of DAG-like molecules. Based on the similar structure of psoralens and khellin, it is possible that khellin acts in the same way as psoralens and that it is functioning along the ET-1 signalling pathway. DAGs are known to activate PKC, and PKC-β in turn has been shown to activate tyrosinase directly by phosphorylating serine residues in the cytoplasmic domain of this protein (Park et. al., 1999). This mechanism of action could explain the increase in tyrosinase activity observed in the present study. These results are further supported by studies which show that tyrosinase activity is increased by phorbol esters, which are also known to mimic DAG-like action (Castagna et al., 1982; Nishizuka, 1984; 1988).

The synergistic action of khellin and UVA remains to be explained. It is possible that UVA causes a photo-modification of the khellin, making khellin a more effective stimulator of the PKC pathway. To test this theory, a number of experiments could be proposed. Firstly, one could test whether khellin treatment does indeed stimulate the release of DAGs and in addition, one could test whether khellin and KUVA result in an increase in the phosphorylation of tyrosinase as described by Park et. al. (1999).

An interesting observation from the present study was that UVA appeared to stimulate the glycosylation of tyrosinase and TRP-2 (see Figures 3.20 and 3.18). Since the glycosylated form is the catalytic (or most active) form of the enzyme *in vivo*, it seems reasonable to suggest that an increase in this form would result in an increase in melanogenesis. This could contribute to the synergism observed after UVA and khellin treatment. Interestingly, western blot results from a study on the effect of psoralen and UVA on melanogenesis in human melanocytes and melanoma cells showed a similar increase in glycosylation in the presence of UVA (Mengeaud and Ortonne, 1996). However, these authors never commented on these interesting results, which warrant further exploration.

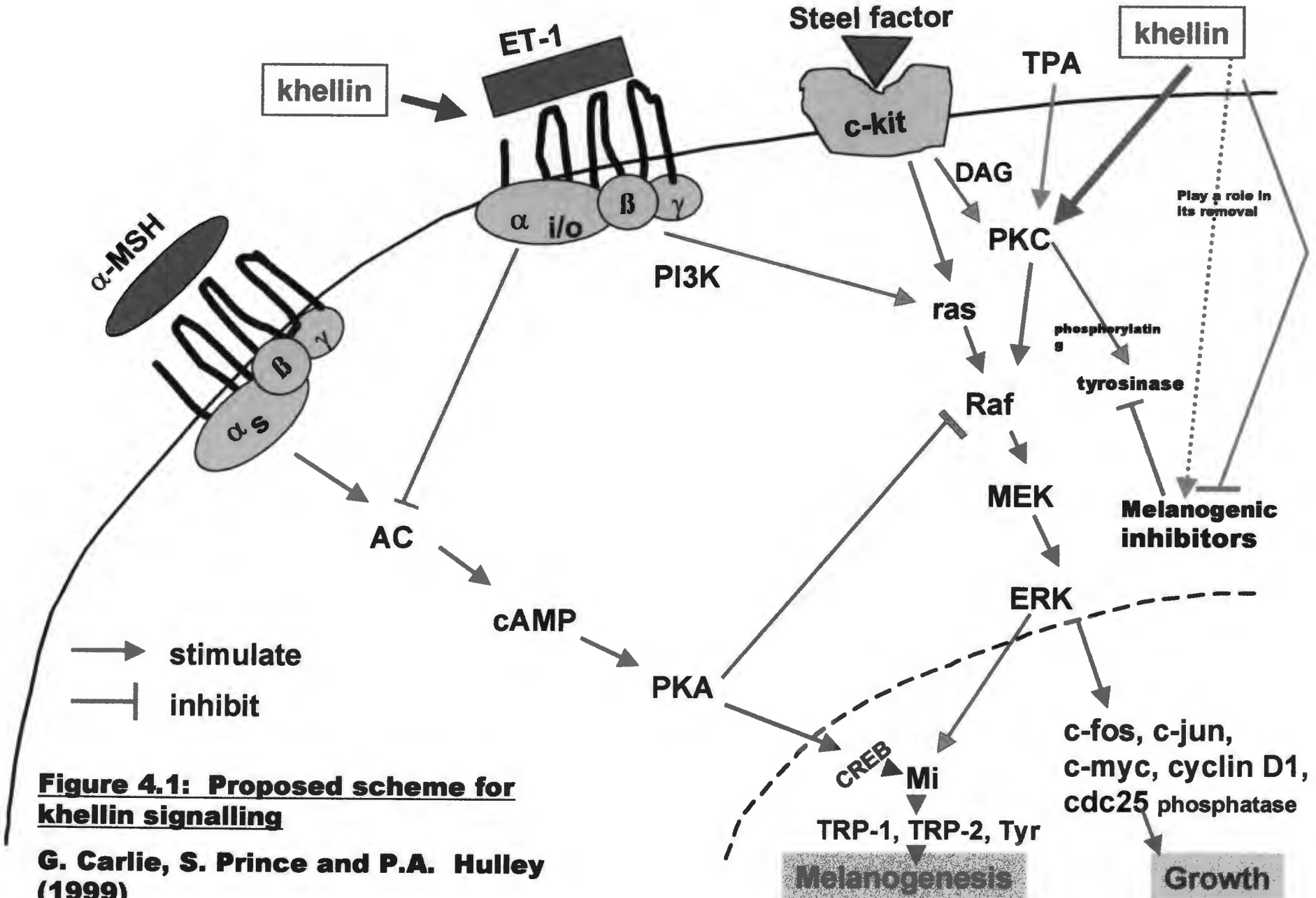


Figure 4.1: Proposed scheme for khellin signalling

G. Carlie, S. Prince and P.A. Hulley (1999)

The long-term effect of khellin and KUVA on melanogenesis

The above discussion has attempted to explain the increase in melanogenesis observed in melanocytes treated for a period of four days with khellin or KUVA. It is also possible that long-term multiple treatments, as would be needed in clinical treatments, would bring about an additional response, similar to the response observed after multiple treatments of melanocytes with UVA during PUVA photochemotherapy (Blog et. al., 1979; Kinley et. al., 1994; Mengeaud and Ortonne, 1996). It is possible that the excision repair mechanism is activated in KUVA-treated cells, and this brings about an enhanced SOS protective response as described for UVB-induced cell damage (Eller et. al., 1994; 1996; Pedeaux et. al., 1998). The activation of p53 tumour-suppressor genes brought about by this response, could in turn directly activate the tyrosinase gene, since tyrosinase promoters contain p53 response elements (see review Ferguson and Kidson, 1997). A further alternative is that via cross-talk between the PKC and PKA pathways, *Mi* is activated (Bertoloto et. al., 1998), and this in turn results in an increase in tyrosinase gene expression. The slight increase in *de novo* forms of tyrosinase and TRP-2 in the western blots of khellin and KUVA treated cells in the present study provide support for this theory. Further support comes from preliminary studies of northern blots of RNA from khellin and KUVA-treated cells, which show a slight increase in *Mi* and TRP-1 mRNA after four days of treatment (Ntusi and Kidson, pers. comm.).

Model for the action of KUVA

The data from the present study allows the following model to be proposed for the mechanism of repigmentation by KUVA. Any successful treatment for vitiligo, must firstly ensure that the depigmented skin lesion is repopulated with melanocytes, which are then stimulated to produce pigment. Transfer of pigment into the surrounding keratinocytes would ensure that skin colour is then restored. With this in mind, the following is proposed:

Stage I: Both khellin and UVA could initiate the repigmentation process by triggering and stimulating the quiescent hair follicle melanocytes and peri-follicular melanocytes to re-enter the cell cycle and begin to proliferate.

StageII: Melanocytes start to migrate into melanocyte-free areas and start to synthesize melanin. Melanin synthesis is stimulated in the short-term by post-translational activation of tyrosinase and possibly, in the long-term, by increased levels of

glycosylated tyrosinase and tyrosinase-related enzymes, and/or by the removal of melanogenic inhibitory substances.

StageIII: In the melanocytes, the level of melanogenesis is increased and the melanocytes begin to transfer melanin to the adjacent epidermal keratinocytes. Continuous treatment with khellin and UVA ensures that melanin synthesis in the migrating melanocytes continues.

Stage IV: Skin colour of vitiligo lesions is restored.

Clinical Implications

An important issue that is brought to light by the present study is the importance of optimizing the topical formulation of khellin. In the clinical trials that were carried out to evaluate the efficacy of the use of khellin by the dermatologists, Honigsman et. al. (1985) and Milne et. al. (1999, manuscript submitted), made up in an aqueous cream base at a concentration of 5% (200mM). Formulation of this khellin cream simply involved mixing the khellin flakes with this aqueous vehicle. A possible criticism of this procedure is that khellin is insoluble in aqueous vehicles, as demonstrated in this study, and thus, the actual concentration delivered to the skin is not known. Furthermore, the concentration of the drug actually delivered to the melanocytes is unknown since nothing is known about the trans-epidermal delivery kinetics of khellin. Nevertheless, it is of importance to note that the topical formulation (200mM) is 20, 000-fold more concentrated than the optimal dose for melanocytes demonstrated in the present study. This clearly highlights the importance of optimizing the topical formulation of khellin and of carrying out drug trials where both the dose applied and the dose delivered are accurately known. With a better cream formulation, the optimal dose could be delivered to the melanocytes and a better repigmenting response might be expected with fewer side effects. In addition, from an economic point of view, if lower quantities of khellin are used in the new cream formulation, it will mean that costs to the patient and the Health Department will be much less. All of these factors should lead to better patient compliance. This is a particularly important consideration in a country like South Africa where many poor patients have to travel long distances for treatment at tertiary hospitals by specialists. Since khellin is safe to use with the natural sunlight that is so abundant in South Africa, these poorer patients will benefit tremendously from an effective treatment that can be used as a home-based treatment, as reported in the study by Milne et al. (1999; manuscript submitted).

Related to the issue of solubility is the question of cytotoxicity. The higher the solubility of khellin

in a vehicle, the higher the dose of khellin that will be delivered to its target cells. The cytotoxicity of khellin at higher doses demonstrated in this study provides an explanation for the ineffectiveness of the topical application of khellin used in a study by Procaccini et. al. (1995), as a treatment for vitiligo. In this study, patients applied khellin on one side of their body either as a 5% cream in an aqueous base or as a 3% cream in 1-methyl-2-pyrrolidinone (PYR), a drug-enhancing vehicle for the dissolution of khellin. On the opposite side of the body, the vehicles were applied as the control before the sessions of irradiation. The results revealed that topical khellin repigmented vitiligo skin, but the khellin-treated patches repigmented no better than those patches treated with vehicle and UVA light only. It would therefore appear that the repigmentation observed in khellin-treated patches was primarily due to the UVA irradiation component of the photochemotherapy sessions.

An explanation for the ineffectiveness of khellin in the aqueous cream formulation in the study of Procaccini and co-workers is possibly due to the poor solubility of the drug in this type of vehicle, as demonstrated in the present study. A consequence of this, is a poor delivery of the drug to the target cells. The absence of repigmentation with PYR is possibly explained by the increase in solubility of khellin in PYR, which may result in higher doses or almost all of the khellin being delivered to the epidermal melanocytes and adjacent cells. A cytotoxic dose of khellin is thus delivered to the cells resulting in their destruction. This probability seems likely since the concentration of khellin used in the study of Procaccini et. al. (1995) was 240-fold more than the concentration of khellin (0.5mM and more) shown to be cytotoxic to both melanocytic and non-melanocytic cells in the present study. Furthermore, an absence of repigmentation could also have resulted from UVA-damage of the skin cells. Procaccini et. al. (1995) suggested that this may have arisen from the emollient action of PYR on the skin, which decreases the barrier effect of the stratum corneum, and thus more UVA light is able to penetrate through the skin layers.

Another question to be considered is the apparent cytotoxic effect of khellin on cultured fibroblasts. One possibility is that the cytotoxic effect on fibroblasts in culture is an experimental artefact and only pertains to *in vitro* studies because fibroblasts grow so much faster *in vitro* than *in vivo*. Fibroblasts residing in the dermis are not highly proliferative and are therefore less likely to be adversely affected by low doses of khellin (especially since this present study demonstrated minimal inhibition). The effect of khellin on dermal fibroblasts will need to be determined. Another concern regarding KUVA treatment is the potentially damaging effects of UVA light on collagen synthesis and turnover. UVA can penetrate into the dermis of the skin because of its long wavelength of light (Abel, 1995). It is therefore possible that UVA treatment could accelerate skin

ageing.

TECHNICAL ISSUES

A technical limitation of the present study was the inability to detect the rate-limiting enzyme of melanogenesis, tyrosinase, in KUVA-treated Mel-1 melanoma cells by western blotting analyses, despite a 200% increase in the melanogenic rate of these cells above the untreated cells. This is possible because the antibody α-PEP 7, recognizes mouse rather than human tyrosinase (V. Hearing, pers. comm.). Davids (1997), has previously demonstrated that tyrosinase is expressed in Mel-1 cells, but at very low levels. For future studies, much longer incubation times with this antibody are possibly needed in order to detect this melanogenic protein in human samples. Alternatively, other antibodies against human tyrosinase and TRP-1 would be helpful.

When the melanogenic assays were carried out, the following useful finding was also made. At the start of this study, the harvested protein from the cultured cells was collected in complete extraction buffer (CEB) and the same sample was then used for both enzyme assays and western blot analyses. Out of interest, a comparison of lysates prepared in CEB with lysates made in phosphate buffer containing 0.5% Triton-X 100 was carried out. The results demonstrated that the incorporation values of samples prepared in Triton-X containing phosphate buffer were almost double the values of those prepared in CEB buffer (Table 1). It is likely that SDS, which is found within the CEB buffer, inhibited the activity of tyrosinase. It was also interesting to note that the phosphate buffer in which protease inhibitors had been added, had lower incorporation values than that found with the Triton-X containing phosphate buffer alone. This suggests that protease inhibitors had an inhibitory effect on tyrosinase activity. It should be noted that the percentage increase in the cpm values above the control, were comparable with the different protein lysis buffers, although the values obtained with Triton-X containing buffer were much higher.

TABLE 1.

	TRITOX – X 100	**TRITON-X 100 WITH $^{\Psi}$P.I**	***CEB**
CPM VALUE +/- S.D.	11518 +/- 1578	7207+/- 891	6541+/- 1921

$^{\Psi}$P.I. = protease inhibitors (see appendix)

*CEB (complete extraction buffer) contains P.I. (see appendix)

LIMITATIONS OF THE STUDY

At the commencement of this present study, the experiments were designed to mimic the *in vivo* treatments as far as possible. Several limitations of this study have been identified and these will be discussed below:

Absence of adjacent epidermal cells: In this *in vitro* study, melanocytes were isolated from other epidermal cells, which are normally present *in vivo*. Thus, the paracrine effects of neighbouring cells were not tested after treatment with khellin or KUVA. As shown in previous studies with PUVA and UV light, both melanocyte proliferation and melanogenesis were increased as a result of these agents acting on adjacent keratinocytes, which responded by increasing the release of melanogenic and mitogenic growth factors (Halaban et. al., 1987; Imokawa et. al., 1992; Abdel-Nasser et. al., 1997). Thus, it is possible that the levels of both proliferation and pigmentation of KUVA-treated melanocytes in this *in vitro* tissue culture system might have been increased even further if co-cultures of melanocytes and keratinocytes were used. Future experiments could therefore be carried out with epidermal constructs, whole skin organ cultures or the melanocytes cultured in KUVA-treated keratinocyte-conditioned medium. In this way, paracrine effects of KUVA on neighbouring cells could be evaluated.

A mixed culture of normal human melanocytes from different racial groups: Since normal human melanocytes from patients of different racial groups were cultured together, it is not possible to make deductions about the responsiveness of melanocytes from different skin types to KUVA treatment. Future studies should include melanocyte cultures from patients with the same skin type.

Single versus multiple UVA irradiations: During KUVA treatment *in vivo*, patients are exposed to multiple irradiations of UVA light or solar irradiation after being treated with topical or oral khellin (Milne et. al., 1999). However, in the present study, the melanocytes and melanoma cells were exposed to a single dose of UVA light, as previously described by Kao and Yu, (1992), who exposed their cultured melanocytes to PUVA photochemotherapy. Mengeaud and Ortonne, (1996), suggested that multiple UVA irradiations were not necessary for stimulating melanogenesis *in vitro* when melanocytes were cultured in the presence of melanocytic mitogens such as TPA (a phorbol ester). Since mitogens were present in the culture medium of the normal human melanocytes, it was decided to use a single UVA dose for this study. In addition, since a melanogenic response was obtained with this single UVA dose, it was decided not to pursue the investigation in which the

effects of multiple UVA irradiations on the melanocytes would be determined. It is quite possible that if multiple UVA irradiation doses were used in the present culture system, more significant differences would have been detected.

Melanocyte migration: It is not known whether the increase in melanocyte proliferation observed after KUVA treatment will necessarily lead to an increase in the melanocyte migration or whether KUVA actually stimulates the migration of these melanocytes directly. An increase in both the processes of proliferation and migration of melanocytes are clearly needed to repopulate the depigmented vitiligo skin to restore skin colour in patients. In this study, however, melanocyte migration studies were not carried out. Future work would therefore include migration studies by means of immunocytochemistry or Boyden chamber assays (Horikawa et. al., 1995), in order to determine whether KUVA treatment affects melanocyte migration.

CONCLUSION AND GENERAL COMMENTS

Khellin alone and in combination with UVA (KUVA), directly stimulates proliferation as well as the pigment-forming ability of both normal human melanocytes and human melanoma cells in culture. Unlike PUVA, KUVA treatment does not rely on paracrine factors from adjacent epidermal cells to bring about these increases in the biological processes of proliferation and melanogenesis. Importantly, the most effective dose of khellin is 0.01mM, which is 20,000 fold lower than the dose currently used (200mM) to make up the cream for clinical trials. Therefore, lower doses than currently employed, combined with a more effective way to dissolve the drug, would possibly be more efficacious than the current treatment regimen. This possibility is supported by the recent clinical trials carried out by Milne et. al., (1999). These investigators demonstrated that more effective repigmentation was obtained with lower khellin doses. This ensures that the optimal dose is delivered to the cells, and therefore adverse effects would be minimized. Since the present study also showed that khellin and KUVA treatment inhibit the proliferation of fibroblasts, this suggests that KUVA acts on signalling pathways exclusive to melanocytes, possibly via the endothelin-1 pathway.

Future studies

- Carrying out drug trials using lower doses of drug in a suitable carrier vehicle. These studies could be combined with histological studies to determine for example, *c-kit* receptor expression

of KUVA-treated skin biopsies.

- Investigating in greater detail the mechanism of khellin action (signalling and downstream events). For example, in order to determine whether ET-1 and KUVA share the same signalling pathway, antagonists for the PKC, PKA and MAP kinase signal transduction pathways could be used.
- Co-culturing of keratinocytes and melanocytes, or melanocyte cultures with keratinocyte-conditioned medium, would provide clues regarding the paracrine effects of khellin and KUVA.
- Testing khellin and KUVA on an animal model for vitiligo (for example *Mitf*^{mit} mutant mouse, Bora et. al., 1999) with an intact epidermal environment and normal immune system. The same model is later then to be used for applying the newly formulated khellin cream. This is to be followed by pharmacokinetic studies, to determine whether the optimal khellin dose is being delivered to the melanocytes. This model will also give an indication of adverse effects likely to be experienced with this cream formulation.

Finally, the information obtained from this cell biological study provides support for more extensive studies to be carried out by dermatologists with KUVA photochemotherapy. This study also emphasizes the need for work on investigating the transdermal delivery of the most effective doses of khellin to the target melanocytes, in view of its potential *in vitro* melanocytoxicity.

APPENDIX

SOLUTIONS:

PHOSPHATE BUFFERED SALINE [PBS] (pH 7.4)

NaCl	8g
di-$Na_2HPO_4.12H_2O$	1.45g
KCl	0.2g
KH_2PO_4	0.2g

pH to 7.4 and made up to one litre with distilled water.

Autoclaved to sterilize.

0.1M PHOSPHATE BUFFER (pH 7.4)

$NaH_2PO_42H_2O$	15.6g dissolve in 1 litre water… solution A
$Na_2HPO_4.2H_2O$	17.8g dissolve in 1 litre water… solution B

Made up solution A and solution B, by dissolving salts with magnetic stirrer bar. Added 200ml solution A to 800ml solution B (1:4) and pH to 7.4.

TISSUE CULTURE REAGENTS:

HAM'S F-10 CULTURE MEDIUM (1 LITRE)

10g	Ham's F-10 powder (Highveld Biological)
1.2g	$NaHCO_3$

pH to 7.2 and made up to 1 litre with distilled water.

Filter sterilize with 0.22µm filter unit

BRL-CONDITIONED MEDIUM

Ham's F-10 culture medium

20% Heat inactivated foetal calf serum

1% Penicillin/Streptomycin (100IU penicillin / 100mg/ml streptomycin)

0.1% Fungizone

SUPPLEMENTED BRL-CONDITIONED MEDIUM (50ml)

48.5ml	BRL-Conditioned medium
1ml	5mM IBMX (Isomethylbutylxanthine)
250µl	TPA (40µM working solution, use 5µl/ml)
100µl	Insulin (100IU/ml)
100µl	Cholera Toxin (2µl/ml)
500µl	Penicillin/Streptomycin (1%)
5µl	Fungizone

HOECHST STAIN 33342: BISBENZIMIDAZOLE DYE

SOLUTIONS:

(1) **ACETIC ACID / METHANOL (1:2)**

Mixed 30ml glacial acetic acid (BDH), with 60ml methanol (Merck)

Hank's Balanced Salt Solution (HBSS) 100ml

HANK'S BALANCED SALT SOLUTION (HBSS) 100ML

Prepared a 10x stock concentrate of HBSS

Solution 1:

8g	NaCl
0.4g	KCL
0.2g	$MgSO_4 7H_2O$
0.09g	$Na_2HPO_4 . 7H_2O$
0.06g	KH_2PO_4
1g	glucose

Dissolved in 80ml distilled water.

Solution 2:

Dissolved 0.14g $CaCl_2$ in 10ml distilled water

Solution 3:

Dissolved 0.02g phenol red in 5ml distilled water

Combined solutions 1 and 3. Added slowly to this, was solution 2 and water to a final volume of 100ml. Chloroform (500µl) was added a preservative.

(3) **HOECHST POWDER STOCK SOLUTION (1mg/ml)**

Weighed 0.001g powder and added 1ml HBSS. Stored stock at 4^0C in dark, covered with foil.

(4) **HOECHST WORKING SOLUTION (1ng/ml) 50ml**

Take 50µl of 1mg/ml Hoechst stock solution, and added 50ml HBSS

(5) **MOUNTING MEDIUM: VERONAL BUFFERED GLYCERINE**

VERONAL BUFFER:

0.51g veronal sodium (barbitone sodium)

0.31g NaCl

Added 40ml distilled water and stirred for 5 minutes with a magnetic stirrer. Made up to 50ml with distilled water. pH to 8.6 with 1N HCL. Thereafter, mixed equal quantities of veronal buffer (pH 8.6) with glycerine (50ml). Stored at 4^0C, until used.

PROTOCOL FOR MYCOPLASMA DETECTION

(1) A day or more before experiment was started, plated cells onto a sterile coverslip within a 35mm^2 dish (Corning). Allowed the cells to reach a density of 60-65% confluency on the coverslip.

(2) Rinsed cells twice with HBSS (2ml for each wash)

Added 1ml HBSS and 1ml acetic/methanol (1:3) solutions to petri dish. Rocked the petri dish and discarded the solution into disposable waste container.

Added 2ml acetic acid/methanol (1:3) solution to the dish. Rocked the dish and discarded the solution.

(5) Added 2ml acetic acid/methanol (1:3) solution to dish and left for ten minutes at room temperature

(6) Discarded acetic acid /methanol (1:3) and washed twice with HBSS

(7) Added 2ml Hoechst working solution (1ng/ml) and left for ten minutes at room temperature

(8) Discarded Hoechst solution into disposable waste tube and into radioactive waste

(9) Rinsed thrice with 2ml HBSS

(10) Placed a drop of veronal mounting medium on to a glass slide and placed the coverslip, with cell side down onto the coverslip.

(11) Left for one hour at 37^0C in a humidity chamber or leave at room temperature overnight.

(12) Viewed under fluorescent microscope for signs of fluorescence, indicative of mycoplasmal contamination.

COMPLETE EXTRACTION BUFFER (CEB)

EXTRACTION BUFFER

10ml	1M Tris-HCl, pH7.2
1ml	100% Nonidet P-40 (nonionic detergent)
100µl	10% SDS (Sodium dodecyl sulphate)
88.9µl	distilled water

Filter sterilized with 0.22µm and stored at 4^0C for six months

CEB PREPARATION

Added the following reagents on ice:

10ml	extraction buffer
10µl	Aprotinin (1mg/ml stock)*
10µl	PMSF (phenyl methylsulfonyl-flouride)$^{\Phi}$

*** (Aprotinin (1mg/ml stock)**

10mg powder added to 10ml sterile distilled water. Aliquotted into sterile Eppendorfs and stored at -20^0C

$^{\Phi}$ (PMSF)

Weighed out 0.087mg PMSF powder and added 5ml isopropanol. Shook until all crystalline powder is dissolved completely. Stored in dark bottle at room temperature on shelf.

PROTEIN ASSAY PROTOCOLS

BCA PROTEIN ASSAY

Pipetted 10µl of sample + 40µl 0.1 M phosphate buffer (pH 7.4) or 50µl standards into Eppendorf tubes in triplicates. 50µl of 0.1 M phosphate buffer was pipetted into the tube as the blank. The standards were prepared from a 2mg/ml stock of bovine serum albumin.

Added 1ml BCA working reagent to each tube and mixed well

Incubated all the tubes for 30 minutes in a 37^0C waterbath.

After incubation, cooled the tubes at room temperature for 5 minutes

Measured the absorbance at 562 nm of each tube against the blank as the reference tube.

Prepared a standard curve by plotting the $A_{(562)}$ reading for each standard versus its concentration in mg/ml. Used the standard curve in order to determine the protein concentration of the unknown samples.

The samples were all standardised to the lowest protein concentration.

BIO-RAD PROTEIN ASSAY PROTOCOL

(i) Prepared several dilutions of protein standards, using 2mg/ml bovine serum albumin as the stock protein solution. All samples were prepared in duplicates.

(ii) Placed 20µl of the diluted sample, having taken into account the dilution factor, and placed 20µl of standards into 2ml sterile Eppendorfs. Placed 20µl of 0.1M phosphate buffer into the "blank" Eppendorf.

(iii) Added 1ml dilute dye reagent (dye: water, 1:50) to each Eppendorf.

(iv) Vortexed or mixed several times by gentle inversion of test tube.

(v) Allowed samples to stand on bench-top for 15 minutes

(vi) Measured samples spectrophotometrically $OD_{(595)}$ versus the blank reagent

(vii) Plotted $OD_{(595)}$ versus the concentration of standards in µg/ml.

(viii) The unknown protein sample concentrations could be determined from the graph.

(ix) The samples were then all standardised to the lowest protein concentration.

^{3}H-THYMIDINE INCORPORATION ASSAY

(i) After the cells were incubated with ^{3}H-thymidine (5µCi/ml), in the presence or absence of khellin for 48 hours, the radioactive medium was aspirated.

(ii) The cells were washed twice with serum-free medium (sfm) or PBS.

(iii) The sfm was aspirated and the cells were washed thrice with ice-cold 10% TCA (trichloroacetic acid, BDH) for 5 minutes per wash, on an ice-tray, to assist the precipitation of the labelled DNA

(iv) TCA was then aspirated in order to remove the free (unincorporated) thymidine.
The cells were washed once with 10% (v/v) SDS for 2 minutes at room temperature to solubilize the cell membranes and release the TCA precipitate.
The solubilized precipitate was placed into 5ml liquid scintillating cocktail and the radioactivity levels were determined with a liquid scintillating counter.

An example of raw data obtained from a typical ^{14}C-tyrosine assay

Khellin concentration (mM)	CPM values obtained with normal human melanocytes exposed to khellin	CPM values obtained with Mel-1 cells exposed to khellin
0	8849	3695
0.01	22855	10177.5
0.1	18848	8095

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